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INTRACELLULAR ENERGETIC UNIT: STRUCTURAL AND FUNCTIONAL ASPECTS

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To Kertu and Paavo with love

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

I Seppet, E. K., Eimre, M., Andrienko, T., **Kaambre, T.**, Sikk, P., Kuznetsov, A. V., Saks, V. Studies of mitochondrial respiration in muscle cells *in situ*: Use and misuse of experimental evidence in mathematical modeling Mol. Cell. Biochem. 256: 219–227, 2004.

II Appaix, F., Guerrero, K., Rampal, D., Izikki, M., **Kaambre, T.**, Sikk, P., Brdiczka, D., Riva-Lavieille, C., Olivares, J., Longuet, M., Antonsson, B., Saks, V. A. Bax and heart mitochondria: uncoupling and inhibition of respiration without permeability transition. Biochim. Biophys. Acta. 1556: 155–167, 2002.

III Seppet, E. K., **Kaambre, T.**, Sikk, P., Tiivel, T., Vija, H., Tonkonogi, M., Sahlin, K., Kay, L., Appaix, F., Braun, U., Eimre, M., Saks, V. A. Functional complexes of mitochondria with Ca,MgATPases of myofibrils and sarcoplasmic reticulum in muscle cells. Biochim. Biophys. Acta. 1504: 379–95, 2001.

IV Saks, V. A.**, Kaambre, T.**, Sikk, P., Eimre, M., Orlova, E., Paju, K., Piirsoo, A., Appaix, F., Kay, L., Regitz-Zagrosek, V., Fleck, E., Seppet, E. Intracellular energetic units in red muscle cells Biochem. J. 356: 643–57, 2001.

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VI Andrienko, T., Kuznetsov, A. V., **Kaambre, T.**, Usson, Y., Orosco, A., Appaix, F., Tiivel, T., Sikk, P., Vendelin, M., Margreiter, R., Saks, V. A. Metabolic consequences of functional complexes of mitochondria, myofibrils and sarcoplasmic reticulum in muscle cells J. Exp. Biol. 206: 2059–72, 2003.

VII Saks, V., Kuznetsov, A., Andrienko, T., Usson, Y., Appaix, F., Guerrero, K., **Kaambre, T.**, Sikk, P., Lemba, M., Vendelin, M. Heterogeneity of ADP diffusion and regulation of respiration in cardiac cells. Biophys. J. 84: 3436–56, 2003.

ABBREVIATIONS

A	actin
ADP	adenosine 5'-diphosphate
AK	adenylate kinase
AMP	adenosine monophosphate
ANT	adenine nucleotide translocase
AP_5A	diadenosine pentaphosphate
ATP	adenosine 5'-triphosphate
BaxFL	full length Bax
BaxDC	C terminal truncated Bax
BKA	bongkrekic acid
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular calcium concentration
$[Ca^{2+}]_c$	cytoplasmic calcium concentration
CAT	carboxyatractyloside
CaMKII	CaM kinase II
CCCP	carbonylcyanide m-chlorophenylhydrazone
CICR	Ca^{2+}-induced Ca^{2+} release
CK	creatine kinase
CsA	cyclosporine A
CPA	cyclopiazonic acid
CyP D	cyclophilin D
DTT	dithiothreitol
DHPR	dihydropyridine receptor
EM	electron microscopy
FAD	flavin adenine dinucleotide
$FADH_2$	1, 5-dihydro-flavin adenine dinucleotide
FCCP	carbonylcyanide-p-trifluorometoxy-phenylhydrazone
Fe-S	iron-sulphur
GTP	guanosine 5`-diphosphate
HREM	high-resolution electron microscopy
ICEU	intracellular energetic unit
IF	intermediate filament
K-Mes	potassium 2-(N-morpholino) ethanesulfonate
LDH	lactate dehydrogenase
M	myosin
MAP	microtubule-associated protein
MARK	microtubule-affinity-regulating kinase
MHC	myosin heavy chain
Mit CK	mitochondrial creatine kinase
MM-CK	muscle creatine kinase

MPT	mitochondrial membrane permeability transition
NAD	nicotineamide adenine dinucleotide
NADH	dihydronicotineamide adenine dinucleotide
NCX	Na/Ca exchanger
OSCP	oligomycin sensitivity conferral protein
OxPhos	oxidative phosphorylation
PBS	phosphate-buffered saline
PCr	phosphocreatine
PEP	phosphoenole pyruvate
PK	pyruvate kinase
PLB	phospholamban
PTP	permeability transition pore
PTPC	permeability transition pore complex
RaM	Ca^{2+} rapid uptake mode
Rh	rhodamine
RR	ruthenium red
RyR	ryanodine receptor
RyR1s	type 1 ryanodine receptor
RyR2s	type 2 ryanodine receptor
SERCA	sarcoplasmic reticulum Ca^{2+} release channel
SR	sarcoplasmic reticulum
TBQ	1,4-benzohydroquinone
TEM	transmission electron microscopy
TG	thapsigargin
TnC	troponin C
TnI	troponin I
TnT	troponin T
TRITC	tetramethylrhodamine isothiocyanate
UQ	ubiquinone
VDAC	voltage-dependent anion channel

HISTORICAL INTRODUCTION

The development of bioenergetics as a science was started more than two and half centuries ago by the works of famous French scientist Antoine-Laurent Lavoisier. He discovered (together with the English scientist Joseph Priestly) that oxygen and the biological oxygen consumption form the basis of the cellular energetic. It took a little less than two centuries until phosphocreatine (PCr) and adenosine5'-triphosphate (ATP) were discovered (Mommaerts, 1969). Karl Lohmann (Lohmann, 1934) showed that ATP and PCr are related to each other by the creatine kinase (CK) reaction. The connection between the oxygen consumption and ATP synthesis was discovered by Vladimir Engelhart, who also found that the contractile protein myosin is an ATPase (Engelhart, *et al*., 1939). The next step in the history was made in 1942, when Albert Sent-Gyorgyi showed directly the connection between muscle fiber contraction and ATP hydrolysis (Mommaerts, 1969). All this information shows that the cellular bioenergetics is a collection of biochemical and biophysical processes in which the production and consumption of ATP plays a crucial role.

Classical bioenergetic research has been usually associated with experimental studies on isolated mitochondria, which play a central role in ATP synthesis through oxidative phosphorylation. These studies have been performed for half a century and were pioneered by Albert Lehninger, Britton Chance and many others. The discovery in 1948 by Eugene Kennedy and Albert Lehninger (Lehninger and Kennedy, 1948) that mitochondria are the site of oxidative phosphorylation in eukaryotes marked the beginning of the modern bioenergetics. Further on Chance and Williams described the phenomenon of respiratory control by ADP for isolated mitochondria (Chance and Williams, 1955; 1956). Peter Mitchell's (Mitchell, 1961) paper introducing the chemiosmotic hypothesis started a revolution in our understanding of the bioenergetics and biologys as a whole and and provided the conceptual basis for understanding a great variety of energy conversion mechanisms including oxidative phosphorylation. In 1993−1994 Paul D. Boyer and John E. Walker identified the rotary mechanism of ATP-synthase reaction (Boyer, 1993; Abrahams *et al*., 1994).

The relatively new but the most important question in cellular bioenergetics is how mitochondria behave in the cells and how they are regulated *in vivo*. It has become evident that information derived from studies on isolated mitochondria *in vitro* is not sufficient to solve this question. *In vivo*, mitochondria are associated with cellular architecture which is broken and lost when we isolate mitochondria from the cells. The results of very numerous studies have also shown that the intracellular medium cannot be taken as a homogenous solution. In living cells there is practically no free protein in solution, even components of glycolytic system are organized into the cellular supramolecular

structures. Not only the macromolecules but also a significant part of the water molecules are not free in the cells (cf. Aliev *et al*., 2002 for review). The use of high-voltage or embedment-free electron microscopy has demonstrated that there exists a very highly elaborate and dynamic cytoskeletal network in the cells which envelope many cellular structures and, most importantly, are linked the mitochondria, with cell membranes, myofibrils and other cellular structures. The highly organized cytoarchitecture results in compartmentation, micro-compartmentation, metabolic channeling, and functional coupling which is the basis of the cell metabolism.

REVEW OF LITERATURE

1. ATP production in mitochondria

1.1. Mitochondria a general description

The mitochondria are membrane-bound organelles, which perform a wide range of functions, including ATP production, fatty acid oxidation, participation in apoptosis, calcium homeostasis, and signaling by reactive oxygen species.

Mitochondria make over 90% of the energy for the cardiac cell use. Models of the mitochondria have come mainly from the transmission electron microscopy (TEM). Palade (Palade *et al*., 1952) and Sjostrand (Sjostrand *et al*., 1953) found that mitochondria have two distinct membranes: the inner membrane and the outer membrane. The outer membrane is smooth and does not contain the folds. The inner membrane, in turn, forms protuberances into the cellular interior known as cristae. The mitochondrial cristae of the inner membrane are the sites of ATP production. The main oxidative mechanism in the cell, the electron transport chain, is also located in the inner membrane of mitochondria.

According to Palade the inner membrane protrudes into the mitochondrial interior in “baffle” like manner and the leaves or lamellae that form the baffle, are aligned more or less orthogonally to the long axis (Fig. 1a). In contrast to Palade´s model the Sjostrand’s model described the cristae as a stack of independent membranous lamellae (septa) and there is no continuity between cristae and peripheral membranes.

Both models are in accordance to the concept of a membranous framework to ordered chains of enzymes. In 1961, when Mitchell presented a chemiosmotic theory of oxidative phosphorylation (Mitchell, 1961), the Palade’s model prevailed. But five years later Daems and Wisse (Daems *e .al*., 1966) reported that cristae are attached to the inner membrane by small tubes called pediculi. Pediculi seemed to be forgotten until 1994, when the new techniques of high-resolution electron microscopy (HSERM) and electron tomography (Lea *et al*., 1994, Mannella *et al*., 1994) showed (Fig. 1b) that tubular cristae predominate in many tissue types in higher animals. Observation that striated muscles contain crista tubes and lamellae in varying proportions, but brown fat contain only lamellar mitochondria, led to the conclusion that that cristae conformation is a direct consequence of the specialized function of the tissue. (Lea *et al*., 1994, Perkins *et al*. 2000). By using scanning electron-microscopic studies (Ogata, *et al*., 1985) and HSERM (Ogata *et al*., 1997) it was found that mitochondrial morphology varied significantly between white, red and intermediate muscle fibers. The white fibers were distinct from the red

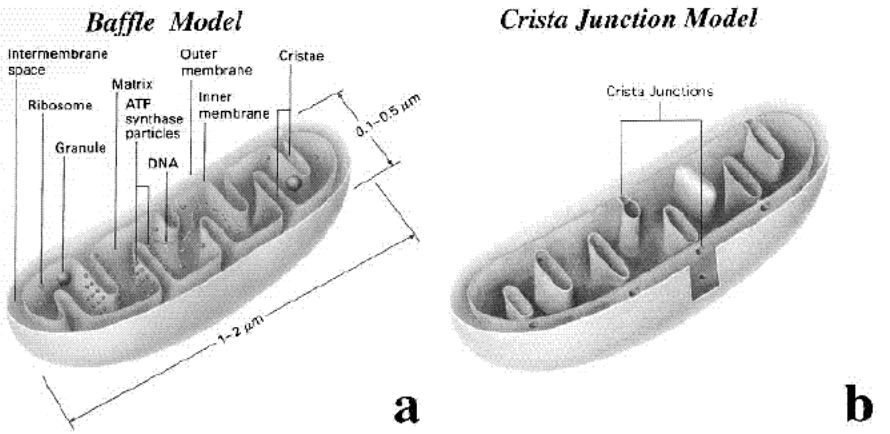


Figure 1. Models of mitochondrial membrane structures. (a) Infolding or "baffle" model. (b) Crista junction model, which supplants the baffle model for all mitochondria examined to date from higher animals. Instead of the large openings connecting the intracristal space to the membrane space present in the baffle model, narrow tubular openings (crista junctions) connect these spaces in this model. (From Perkins, *et al*., 2000, with permission)

and intermediate fibers by their paired long, thin mitochondria, which enriched several myofibrils at the I-band level. Structural features of mitochondria in red and intermediate fibers were similar, but red fiber mitochondria were stubbier, and were connected by a slender stalk across the A-band to the next row of mitochondria. Mitochondria in intermediate fibers were thinner and longer and possessed a slenderer stalk. Contacts between outer and inner boundary membranes have been reported in all types of mitochondria and they are grouped into four types: (i) morphological contacts visualized by electron microscopy, (ii) contacts for channeling metabolites from the matrix to the cytoplasm and *vice versa*, (iii) contacts coordinating fusion and fission events of mitochondria, and (iv) contacts that are involved in translocation of proteins (Reichert and Neupert, 2002).

1.2. Respiratory chain and formation of the inner membrane potential

Aerobic organisms synthesize ATP mainly by two means: by glycolysis in the cytosol and by oxidative phosphorylation (OxPhos) on the inner mitochondrial membrane. OxPhos comprises a respiratory chain with three proton pumps (NADH dehydrogenase-complex I, cytocrome *c* reductase or cytochrom bc_1-

complex III, and cytochrom c oxidase-complex IV) and an enzyme complex not pumping protons (succinate dehydrogenase-complex II) (Fig. 2–4). Respiratory chain complexes pump protons across the inner mitochondrial membrane to create proton electrochemical potential (Δp) called also proton motive force as defined by Mitchell (Michell, 1961; 1966). The proton motive force consists of an electrical (Δψ) and chemical part (ΔpH).

The energy released by the transfer of electrons from respiratory substrate to oxygen is coupled to the translocation of protons from the matrix side to the external side of the inner mitochondrial membrane at thee sites: respiratory chain complexes I, III and IV as noted above. In intact, well-coupled mitochondria the inner membrane is relatively impermeable to the back flow of the protons.

Uncoupling agents (dinitrophenol, carbonylcyanide m-chloro phenylhydrazone (CCCP), carbonylcyanide-p-trifluorometoxy-phenylhydrazone (FCCP) and fatty acids) abolish the obligatory linkage between the respirator chain and the phosphorylation system resulting in back flow of the protons into the matrix (proton leak) (Fig. 2.).

The stoichiometric efficiency of OxPhos is defined by the P/O ratio, or the amount of inorganic phosphate incorporated into ATP per amount of consumed oxygen molecule. The oxidation of dihydronicotineamide adenine dinucleotide (NADH), 1, 5-dihydro-flavin adenine dinucleotide ($FADH_2$) and ascorbate by O_2 are associated with synthesis of three, two and one ATP molecules, respectively. Maximal P/O ratio for NAD-linked substrates is 2.5–3 and 1.5–2 for succinate (Lee *et al*., 1996a, Hinkle, *et al*., 1979). Uncoupling of OxPhos describes any process which decrease the Δp (Kadenbach, 2003), thus leading to "waste" of energy and increased thermogenesis. The compounds which could degrade the proton gradient and decrease the P/O ratio are known as uncouplers of OxPhos (Heytler 1980; Terada 1990), that induce nonspecifical proton leak through the inner mitochondrial membrane (Terada, 1990). Some of the typical uncouplers are listed in Fig. 2.

1.3. Mitochondrial ATP-synthase

The F_oF_1-ATP synthase (Fig. 5) is an ubiquitous enzyme which couples proton flow to ATP hydrolysis or synthesis. The enzyme is composed of two moieties: the F_o is embedded in the membrane as an integral part of the inner mitochondrial membrane and mediates proton translocation into the mitochondrial matrix and the F_1 protrudes from the membrane into the matrix and serves as the catalytic center of the ATP synthesis. The rotary mechanism of F_oF_1-ATP synthase has been proposed independently by Boyer (Boyer and Kohlbrenner, 1981) and Cox (Cox *et. al*., 1984). While Boyer developed the

ATP-synthase binding change principles for ATP synthesis on F_1 and Cox suggested a principle for proton transport in F_o of the ATPase (Boyer, 1993; 1997, Cox *et. al*., 1984).

The bovine complex contains 16 different proteins (including the regulatory subunit F_1) with total a mass of ~600 kDa (Walker *et al*., 1991). The extramembranous F_1 catalytic subcomplex is attached to the F_o subcomplex in the membrane by central peripheral stalks (Walker, 1998). As protons pass through the membrane domain, a rotational force is generated in Fo between the ring of c-subunits and the a-subunit that causes the entire central stalk, a rotor- the $\gamma\delta\varepsilon$-c_{10} subcomplex-to turn (Sambongi *et al*., 1999; Stock *et al*., 1999; Panke *et al*., 2000) and drives the synthesis of ATP in the $\alpha_3\beta_3$ subcomplex of F_1. The enzyme can also catalyze the reverse reaction, with ATP hydrolysis in F_1 driving proton pumping in F_o.

The asymmetric central rotor of ATP synthase has the ability to rotate 360° relative to the stator. Consequently, a population of ATP synthase assemblies is likely to contain a heterogeneous mixture of conformations of the inherently asymmetric F_1 region and a variety of positions of the central rotor with respect to the stator (Rubinstein *et al*., 2003). There are two energy requiring steps in the ATP synthesis: the P_i binding and the ATP release (cf. Al-Shawi, 1990). Binding of substrates and release of the product were considered to be energy linked steps whereas the chemical step was thought to occur without the free energy change. Boyer proposed that three catalytic sites pass sequentially through three different conformations linked to subunit rotation and at any moment in time different steps of the enzymatic mechanism are occurring at each of the three sites.

Evidence now strongly supports the trisite mechanism. The trisite mechanism (Fig. 6) claims that filling of one or two sites are not enough for rotation, and only the filling of the third catalytic site brings about the conformational changes and catalytic cooperativity that are critical for operation of the mechanoenzymatic mechanism. (Weber and Senior, 2003)

At low ATP concentrations the rotary motor rotates in discrete 120^o steps. Each 120^o step consists of 90^o and 30^o substeps, each taking only a fraction of a millisecond (Yasuda *et al*., 1998). This process was investigated by viewing the reaction in the direction of hydrolysis, but it supports the binding-change model for ATP synthesis.

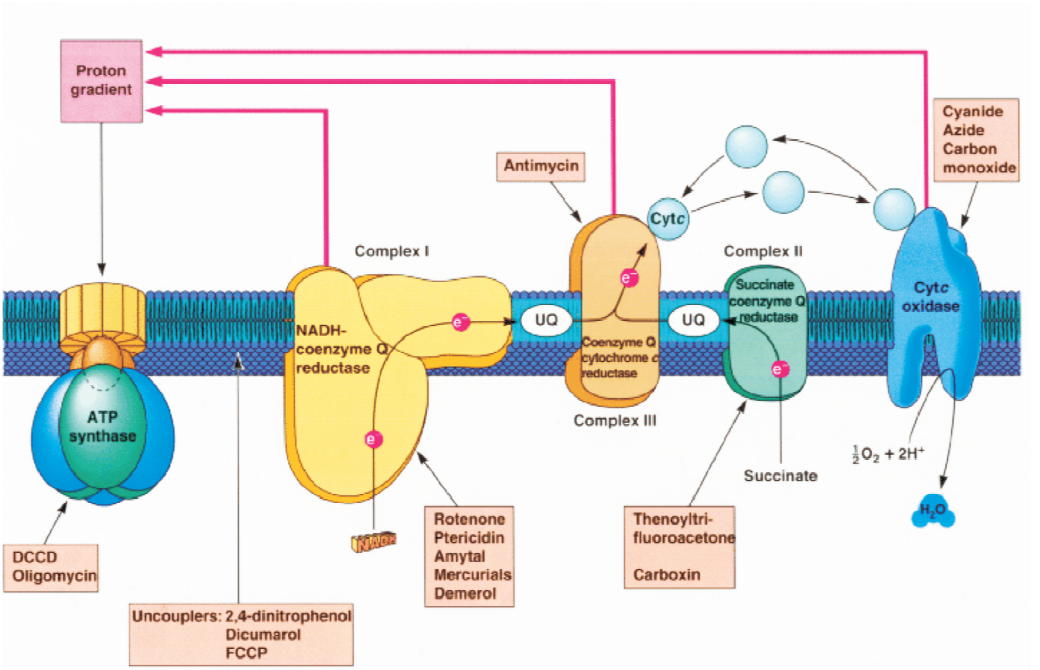


Figure 2. **Electron transport and the respiratory chain** Electrons from NADH pass trough the complex I, the large enzyme complex composed of 42 polypeptide chains including at least six iron-sulphur centers (Fe-S), to the ubiquinone (UQ). Amytal, rotenone, piericidin A, demerol and mercurials inhibit the electron flow from the Fe-S centers of Complex I to ubiquinone. Complex II–succinate dehydrogenase is smaller and simpler than Complex I. It contains at least four different. Electrons pass from succinate to FAD, then through the Fe-S centers to ubiquinone. Complex II can be inhibited by carboxin and thenoyltrifluoroacetone. Complex III (also called cytochrome bc_1 complex) is a dimer of identical monomers, each with 11 different subunits and couples the transfer of electrons from ubiquinol (the fully reduced form of ubiquinone) to cytochrome c with the vectorial transport of protons from the matrix to the intermembrane space (inhibitor antimycin A). The final step of respiratory chain is Complex IV-cytochrome oxidase that carries electrons from cytochrome c to molecular oxygen, reducing it to H_2O. Complex IV is a large enzyme of the inner mitochondrial membrane. The well-known poisons-cyanide, azide and carbon monoxide-are the inhibitors of cytochrome oxidase. (From Garrett and Grisham, 1995, with permission)

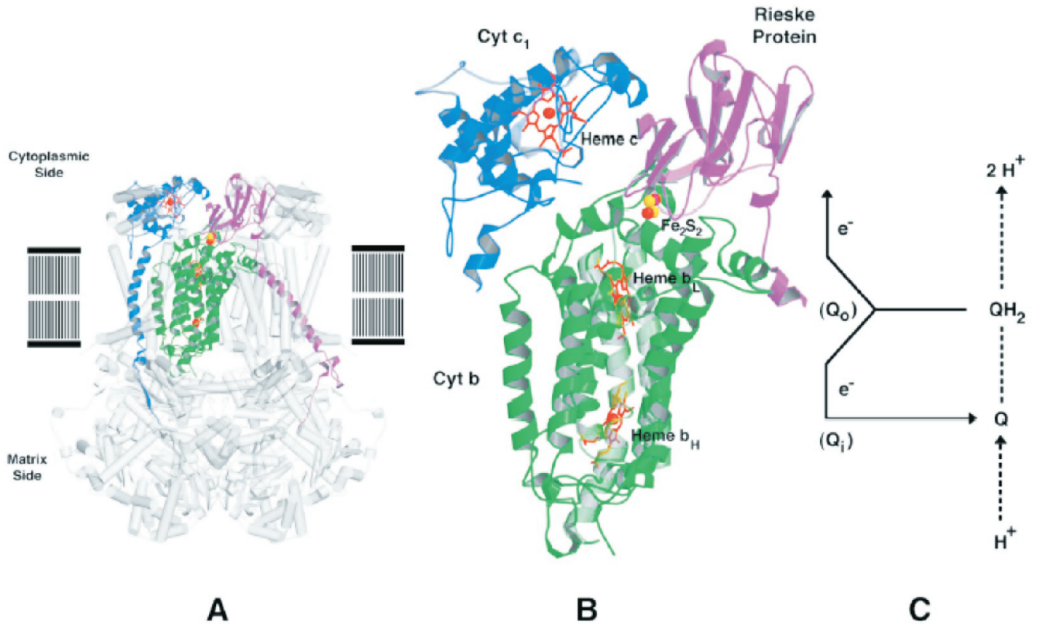


Figure 3. Cytochrome bc1 and the Q cycle. (**A**) Structure of the bovine mitochondrial cytochrome bc1 deduced from x-ray crystallography. Cytochrome bc1 is a stable dimer, with the total molecular mass of the monomer 240 kDa that delivers electrons from ubiquinol to cytochrome c. It couples this redox reaction to the generation of a proton gradient across the membrane by a mechanism known as the Q cycle. This requires two active sites, one for the oxidation of ubiquinol and release of protons on the outer surface of membrane (Qo), and the other for the reduction of ubiquinone coupled to the uptake of protons from the inner side of the membrane (Qi). This mechanism requires that electrons be transferred from the Qo site to the Qi site (**B**) The three subunits that form the functional core of the enzyme are cytochrome b (green), the Rieske ISP (Fe_2S_2 ,purple) and cytochrome c1 (blue). The Fe-S center in this structure is close to the Qo site; it moves toward cytochrome c1 after reduction. The two hemes of cytochrome b have different redox potentials. The Qo site is near the b_L heme (low potential), and the Qi site is near the b_H heme (high potential). (**C**) The topology of electron and proton transfer in the Q cycle mechanism. Bifurcation of electron transfer occurs in the Qo site. A quinol can donate two electrons. The electron transfer within the cytochrome bc1 complex is bifurcated such that the first electron is transferred along a high-potential chain to the Rieske Fe-S center, and then to cytochrome c1, which delivers it to the soluble cytochrome c. The second electron is transferred to the Qi site via the hemes b_L and b_H of the cytochrome b subunit. This is an electrogenic step (it creates part of the protonmotive force) that is driven by the difference in redox potentials of the two hemes (From Saraste, 1999, with permission).

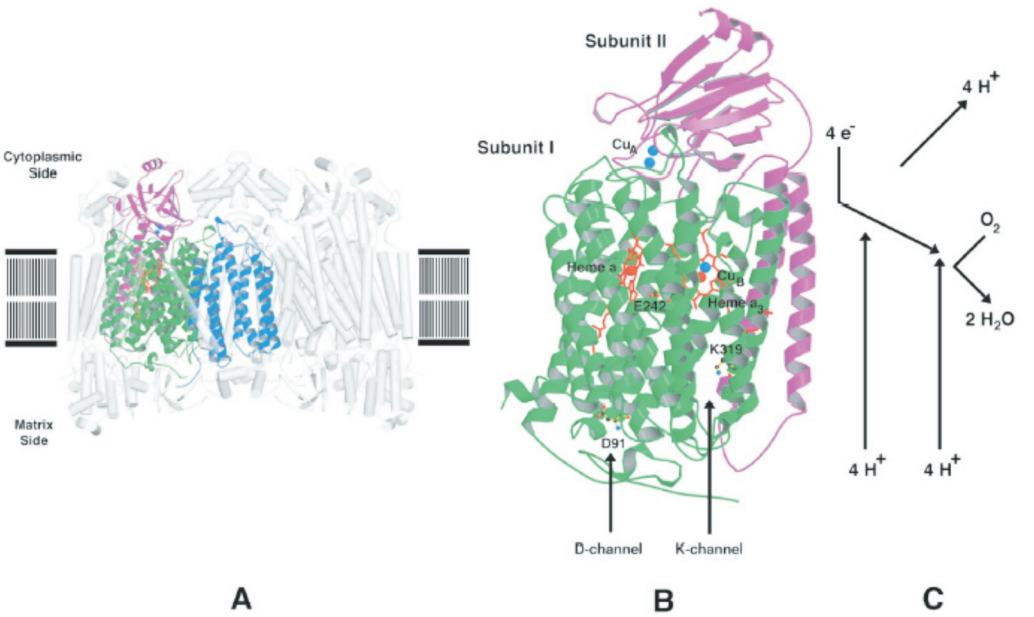


Figure 4. Cytochrome oxidase. (**A**) Structure of the dimeric bovine cytochrome c oxidase deduced from x-ray crystallography. The monomer consists of 13 subunits (total molecular mass 204 kD). (**A**) Subunits I (green), II (purple), and III (blue) are encoded within the mitochondrial genome and form the functional core of the enzyme. (**B**) Subunits I and II contain the metal centers. The active site (cytochrome a3/Cu_B) resides in subunit I. Cytochrome c binds to the cytoplasmic side of this complex, and electrons are transferred to the active site via Cu_A and cytochrome a. (**C**) The topology of electron and proton transfer in cytochrome oxidase. Protons that are used to reduce O_2 into water or pumped to the cytoplasmic side of the mitochondrial inner membrane are transferred through two channels (D and K) from the matrix side. This two hydrophilic channels connect the active site to the aqueous phase of the mitochondrial matrix. These channels are called D and K after a conserved aspartate and lysine, respectively. A conserved glutamate (E242) in the middle of the membrane, at the end of the D channel, is essential for proton pumping activity (From Saraste, 1999, with permission).

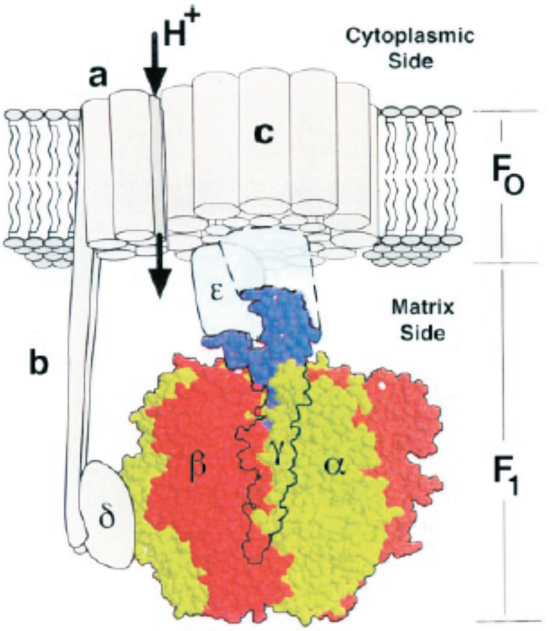


Figure 5. A model of the ATP-synthase. The membrane sector (F_o) contains an oligomer of subunit c that rotates when protons move from the cytoplasmic side of the membrane to the mitochondrial matrix .Each subunit c contains a conserved carboxylic acid residue in the middle of the membrane bilayer. A dodecamer of subunit c is connected to a complex of subunits γ and ε, forming the rotor. Subunit a, a dimer of subunit b, and subunit δ form the stator arm, which has an interface with the oligomeric subunit c and links with the F_1 head. A key component of the stator is subunit a, which contains a conserved arginine that could counteract the moving glutamate in subunit c. The active sites are present in the three β subunits. The three catalytic sites are located at the ab interfaces, and the proton channel is at the c-a interface. (From Wang and Oster, 1998, with permission).

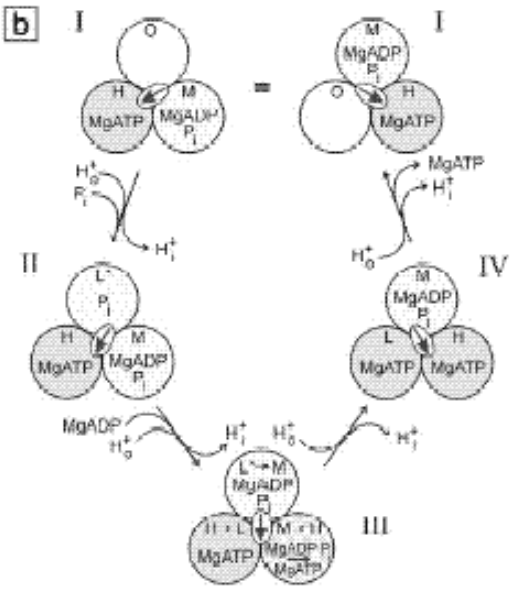


Figure 6. Enzymatic trisite mechanisms of ATP synthesis. Catalytic site conformations are: O, open (unoccupied); H, highest affinity for nucleotide; M, medium affinity; L, lowest affinity; L*, site with Pi binding pocket present. The central arrow denotes Q-subunit rotation. Proton-driven Q rotation generates L* from O (ICII) so that Pi binds. This allows discrimination so that ADP binds (IICIII) despite an unfavorable [ATP]/[ADP] ratio in the cell. Next the binding change occurs (IICIIICIV) and ADP+Pi condense chemically at the (new) H site. Release of ATP involves transformation of an H site via L to O site (IIICIVCI). (From Weber and Senior, 2003, with permission)

1.3. Mitochondrial carrier family and voltage-dependent anion channel

Inner mitochondrial membrane contains a family of proteins (mitochondrial carrier family, MCF), which function is to transport multiple substrates into mitochondrial matrix and export metabolites. Among them is the adenine nucleotide translocase (ANT), a phosphate carrier, uncoupling protein (UCP) and multiple transporters of Krebs cycle metabolites (Aquila *et al*., 1987, Garlid *et al*., 2000). The outer membrane contains single voltage-dependent anion channels (VDAC) for communication between mitochondria and cytosol.

1.4.1. Adenine nucleotide translocase

ANT (also called ADP/ATP carrier) is a key energetic link between the mitochondria and cytosolic energy transport compartments. This antiporter is responsible for the transmembrane exchange between ATP generated inside mitochondria by OxPhos and cytosolic ADP. ANT consists of about 300 amino acids and is located in the inner mitochondrial membrane: its abundance amounts to 10% of the inner membrane proteins in mitochondria from tissues with high-energy requirements (Klingenberg, 1969; Kramer *et al*., 1989; Klingenberg, 1979). ANT is tightly associated to six molecules of cardiolipin (Beyer and Klingenberg, 1985) which is required for the nucleotide translocation (but not for binding) (Hoffmann *et al*., 1994).

Based on analytical centrifugation and neutron diffusion experiments performed in the presence of specific inhibitors, ANT is considered to be organized into homodimers (Block *et al*., 1982; Hackenberg and Klingenberg, 1980). In contrast, biochemical data support the hypothesis that the functional form of ANT would be a homotetramer, each dimer being involved in the transport of one nucleotide, ATP or ADP (Brandolin *et al*., 1985; Fioret *et al*., 1998).

Nucleotide exchange between cytosol and mitochondrial matrix follows strict stochiometry of 1:1, i.e. for one imported ADP one ATP is exported through the mitochondrial inner membrane. The ADP/ATP exchange follows the Michaelis-Menten kinetics, with a *K*m of 1–10 μM for ADP and a *K*m of 1–150 μM for ATP for rat liver ANT (Fioret *et al*., 1998). During the aerobic energy metabolism, these two nucleotides compete for transport, which is not electroneutral, ATP possessing four negative charges and ADP three negative charges. As a result of the mitochondrial transmembrane potential, ATP is preferentially exported from the matrix. The transport of adenine nucleotides is associated with significant conformational changes within each monomer of ANT, as it has been revealed recently by structural analysis at 2.2Å resolution by X-ray crystallography (Pebay-Peyroula *et al*., 2003).

ANT is expressed as three isoforms (ANT1, ANT2 and ANT3). In man, cattle and mouse ANT1 isoform is predominantly expressed in cardiac and skeletal muscles, ANT2 is ubiquitous, being expressed in all tissues in variable amounts depending on the respiratory activity of the tissue. The ANT3 is expressed at a lower level, if at all, in brain, liver, kidney, heart and skeletal muscle.

For the studies of nucleotide transport, two types of highly specific and effective inhibitors of ANT are used (Fig. 7). Carboxyatractyloside (CAT) binds to the ANT from the outside of the inner membrane, whereas the bongkrekic acid (BKA), another inhibitor, preferentially binds from inner side of the membrane.

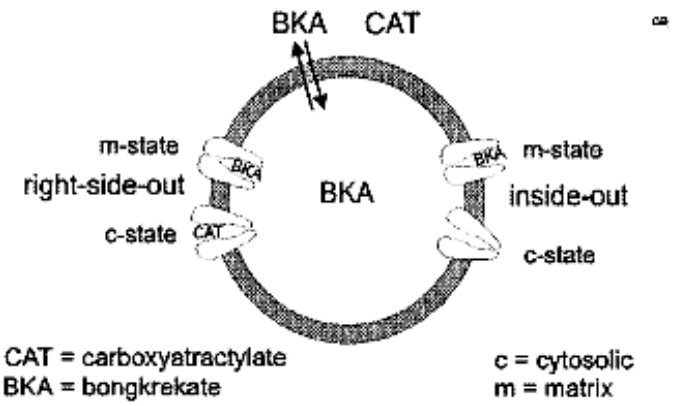


Figure 7. The inhibition of ANT by CAT and BKA. BKA can permeate the membrane and inhibits the carrier in the matrix state (m-state), whereas CAT cannot permeate the membrane and inhibits the carrier in the cytosolic state (c-state). (From Gropp *et al*., 1999, with permission)

The ANT appears to act as a bifunctional protein, which, on one hand, contributes to the ADP/ATP translocation, but on the other hand, can be converted into a proapoptotic pore. Therefore ANT may play a significant role in the permeability transition pore (PTP) complex not only at the structural level in the contact site, but also at the functional level in the regulation of mitochondrial membrane permeability during apoptosis (Belzacq *et al*., 2002).

1.4.2 Mitochondrial porins. Voltage-dependent anion channel

The common pathway for the translocation of metabolites through the outer mitochondrial membrane is the VDAC (Colombini, 1979; Xu *et al*., 1999). Porins are present in the cell of most organisms from *Esherichia coli* to potato and primates. This channel protein is a major mitochondrial outer membrane protein, comprising as much as 60% of the total outer membrane proteins (Thinnes *et al*., 1990). The pore-forming protein is relatively small; its molecular weight is about 30 kD (Mannella *et al.,* 1975; Mannella, 1982). Porins form channels from sided β-sheets. According to different studies, between 12 to 16 β-sheets have been recognized in porin molecules. Mitochondrial outer membrane pore form a β-barrel composed of 16 strands as well.

The selectivity of porins towards anionic species depends on the size and direction of membrane potential. As shown in experiments with reconstituted phospholipid membranes, the channels are open for anionic compounds such as Cl^-, Pi, and adenine nucleotides (Colombini, 1979; Colombini, 1994) at

voltages smaller than 20−30 mV (Vyssokikh and Brdiczka, 2004). At a voltage smaller than 30 mV (high conductance state) the pore has a diameter of 4 nm. Above 30 mV (low conductance state) the diameter of the pore is reduced to 2 nm. The voltage-dependent conductance variations are certainly linked to structural modifications of VDAC. Macromolecules, which cannot penetrate the pore (dextran), have an osmotic effect on the aqueous interior of the channel and by that increase voltage sensitivity. In the presence of dextran the low conductance cation selective state is adopted already at 10 mV (Vyssokikh and Brdiczka, 2004).

The mammalian VDAC gene family consists of three isoforms (VDAC1, VDAC2 and VDAC3), each of which shares approximately 70% sequence identity with the other two family members. In human, there have been found five porin coding genes but only three isoforms are expressed (Decker and Craigen, 200; Winkelbach *et al*., 1994). Knockout of just one of the 3 VDAC isoforms in mice causes serious effects but no single isoform is required for cell viability (Wu *et al*., 1999). The knock-out of VDAC3 in mice resulted in male sterility due to non-motile sperm (Sampson *et al*., 2001) while the knock-out of either VDAC1 or VDAC2 yields mice with a 30% reduction in respiratory capacity (Wu *et al*., 1999). The lack of VDAC1 results in embryonic death of some mice but more frequently embryonic death occurs in mice lacking both VDAC1 and VDAC3 (Colombini, 2004). The absence of VDAC1 in skeletal muscle of a child with a severe mitochondrial encephalomyopaty suggests that VDACs have an important regulatory role in energy metabolism (Huizing *et al*., 1996).

It was thought for a long time that the role of porin channels in the mitochondrial outer membrane is only to limit the dislocation of high molecular weight compounds (>3–10 Da) (Benz, 1990). This understanding is changed now as it has been described in many studies that regulation of the porin channels play important role in ATP production and mediation of the transport of adenine nucleotides (Liu and Colombini, 1992; Hodge and Colombini, 1997; Vander Heiden *et al*., 2000).

A soluble protein called the VDAC modulator isolated from mitochondria has been found to modulate the permeability of VDAC. This protein was first found in *Neurospora crassa* and further discovered in species from other eukariotes. The role of this modulator is still unclear (Liu and Colombini, 1992; Liu *et al.*, 1994). In some tissues (liver, brain) VDAC also acts at the mitochondrial periphery as a binding site for enzymes such as hexokinase (Fiek *et al*., 1982; Nakashima, 1989) and glycerol kinase (Ostlund *et al*., 1983, Towbin, 1989), this indicating the possible connections of the porin channel function to the glycolytic ATP synthesis. In the presence of NADH porin channel permeability is limited for ADP (Lee *et al*., 1994; Lee *et al.*, 1996b). This ability of NADH to facilitate VDAC closure could be one mechanism by which glycolysis can suppress oxidative phosphorylation (Crabtree effect) (Sussman *et al.*, 1980).

The fact that porin channels bind strongly ATP with stochiometry 1:1 indicates also that porin channels regulation may plays important role in energy metabolism (Rostovtseva and Bezrukov, 1998).

VDAC is associated with the initiation of the mitochondrial phase of apoptosis (Bernardi *et al*., 2001; Vander Heiden *et al.*, 2001). Some researchers have linked VDAC to the structure of the permeability transition pore, but others have proposed (Halestrap, 2002) that VDAC may modulate, but is not structurally involved in the permeability transition pore.

2. ATP consumption

2.1. Contractile apparatus and contractile cycle

2.1.1. Myofilaments

The myofilaments of the contractile machinery of the cell are responsible for transducing chemical energy into mechanical energy and work. They occupy 45–60% of the cell volume in mammalian ventricle (Bossen *et al.*, 1978; Forbes and Sperelakis, 1995; Page, 1978.).

Myofilaments are composed of the thick (myosin) and thin (actin) filaments as well as associated contractile and cytoskeletal components (Fig. 8). The functional unit of skeletal muscle is a sarcomere which borders are formed by Z-lines. Sarcomeres consist of parallel arrays of ~1.0 μm long thin filaments that interdigitate with laterally aligned thick filaments. Therefore, in myofibrils composed of many sarcomeric units, the titin filaments form a contiguous filament system. There are three to six titin molecules associated with each thick filament in each half sarcomere in vertebrate muscle (Squire, 1997) Titin interacts directly with several thick filament-associated proteins (myosin-binding protein C (yellow transverse lines in Fig. 8), myosin binding protein H, M-protein, myomesin and the rod region of myosin heavy chain). Titin also interacts directly with actin filaments near the Z-lines and with the Z-line components α-actinin and T-cap (telethonin; dark blue in Fig. 8). Through its interaction with titin, α-actinin crosslinks the third filament system with the thin filaments; these interconnections probably help to transmit forces between adjacent sarcomeres. The N-terminal regions of titin molecules from opposite sarcomeres overlap in the Z-lines, and the C-terminal regions of titin molecules from opposite half-sarcomeres overlap in the M-lines. The thick myosin filaments are ~ 1.6 μm long and 15 nm thick. Each thick filament is composed of ~300 myosin molecules. Myosin heads (known as myosin ATPase) protrude from the long axis after every 14.3 nm, with the protrusion angle rotating 120 ^{o}C at each point. These myosin heads create the crossbridges that interact with actin to generate the contraction. When the cytoplasmic [Ca^{2+}] rises, the myofilaments are activated in a Ca^{2+}-dependent manner, thereby transducing the chemical energy (ATP) into mechanical force of shortening.

2.1.2. The contraction of striated muscle

Contraction of striated muscle is based on the interaction of the myosin head (S1) with actin, and is powered by the coupled hydrolysis of ATP. During the cross-bridge cycle, actin (A) combines with myosin (M) and ATP to produce force, adenosine diphosphate (ADP) and inorganic phosphate, Pi.

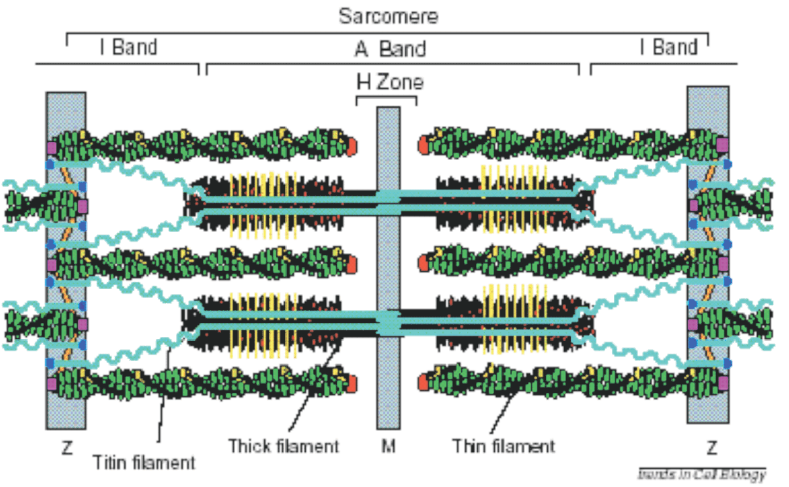


Figure 8. Major components of a cardiac muscle sarcomere: a single contractile unit. The thin filaments are bound at the Z-lines and the center of the thick filaments is known as the M-line. The I-band (isotropic band) is the area where there are only thin filaments and the A-band (anisotropic band) is determined by the length of thick filaments. Rod-like tropomyosin molecules (Tm) (black lines) are associated with each other head to tail, forming two polymers per thin filament; the polymers stabilize the thin filaments. Each tropomyosin molecule binds one troponin (Tn) complex (composed of troponins T(TnT), I (TnI) and C (TnC); yellow). The third filament system is formed by titin, the largest protein yet identified in vertebrates, at 30003700 kDa. Single molecules of titin reach from the Z line to the center (M line) of the sarcomere. This molecule centers thick filaments in the sarcomere and acts as an elastic spring element during muscle contraction. (From Gregorio and Antin, 2000 with permission)

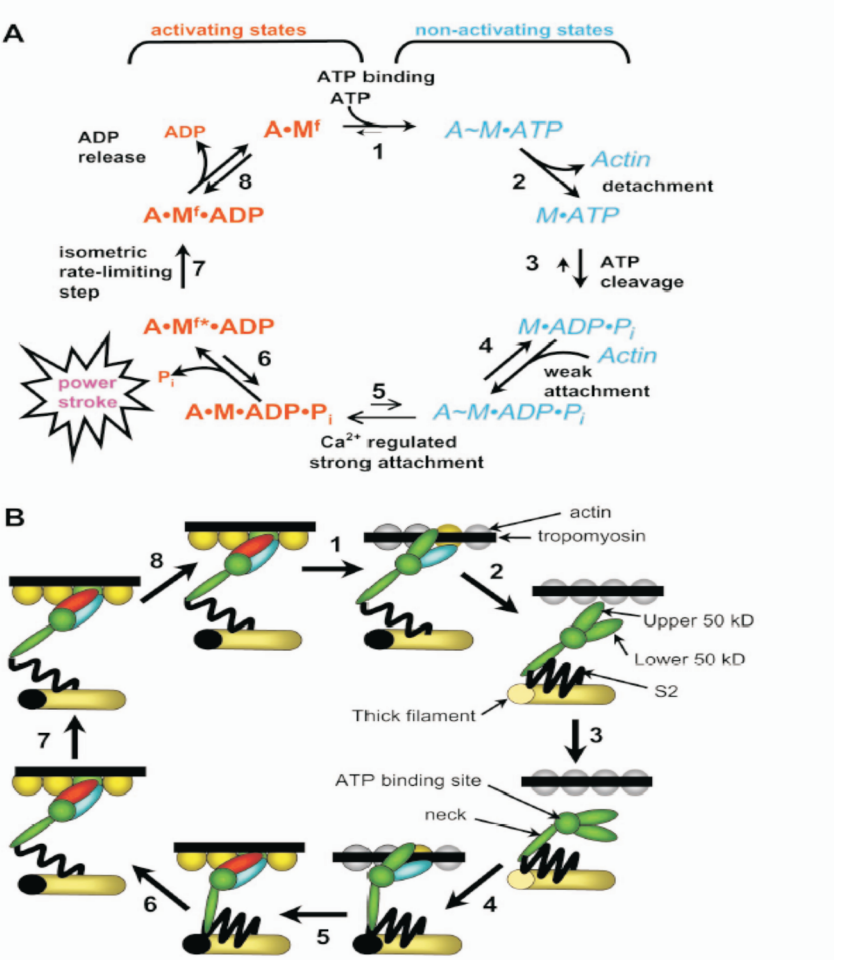


Figure 9. The mechanism of the cross-bridge cycle. ATP binding to myosin (step 1) is very rapid and irreversible. The subsequent detachment of actin from the actin-myosin·ATP (A~M·ATP) complex (step 2) is similarly rapid and is caused by an opening between myosin's upper and lower 50-kDa regions (Fig. 9B) like the opening of jaws. A "flexing" or bending of the myosin neck region (step 2 in Fig. 9B) is followed by step 3, the hydrolytic cleavage of ATP. Following ATP cleavage, myosin again binds weakly to actin at a high rate, but in the absence of Ca^{2+} Tm sterically blocks access of the myosin head to strong binding sites on actin (Fig. 9B). However, when Ca^{2+} is bound to TnC, TnI detaches from actin, allowing the Tm/Tn complex to roll or slide over the thin filament surface. Consequently, the rate of strong cross-bridge attachment, the flux through step 5, is dependent on [Ca^{2+}] and Tm position. Strong binding of myosin to actin (Fig. 9B) is associated with movement of the upper and lower 50?kDa subdomains toward each other (or closing the jaws). In any event, myosin neck extension related to the release of Pi, step 6, is the power stroke that, in isometric muscle, stretches an elastic element (represented here as the S2 segment) by some 10 nm and produces a force of ~2 pN/cross-bridge (Molloy *et al*., 1995). In nonisometric conditions, shortening of the neck extension causes the thick and thin filaments to slide past each other. Step 7 is an irreversible isomerization and is the rate-limiting step for the cross-bridge cycle. Finally, ADP is released from A·M^{f}·ADP (where f is a cross-bridge exerting force) in the reversible step 8 to form the rigor state, A·M^{f}. Noninteracting cross-bridge-actin pairs are shown as gray actin and green myosin, weak interactions as yellow actin and light blue myosin, and strong interactions as green and red myosin, elastic element is represented as the S2 segment (From Gordon *et al*., 2001, with permission).

A + M + ATP→A + M +ADP +Pi + force (1)

A physical model of this transduction known as the sliding filament theory came from x-ray diffraction studies (Huxley, 1969) and mechanical perturbation studies (Huxley and Simmons, 1971). The chemical steps involved in the cross-bridge cycle have also been extensively characterized and correlated with the physico-mechanical schemes (Goldman, 1987; Brenner, 1987). Although the cycle is the same for skeletal and cardiac muscle, the rate constants controlling cross-bridge intermediate transitions are different

Figure 9 shows the cross-bridge cycle in terms of the various reactants and products (9*A*) and the corresponding structural changes (9*B*). Cross-bridges attach and exert force constantly during steps 7, 8, and 1 during isometric contraction, and force drops to zero when the cross-bridges detach in step 2. During isotonic shortening contractions the filaments slide over each other, the strain on the cross-bridge is reduced, and step 7 occurs more rapidly. This accounts for the Fenn effect (an increased rate of energy liberation above the isometric rate as shortening velocity increases). The chemomechanical mechanism shown in Fig. 9 implies that during an isometric contraction, a cross-bridge remains strongly attached to actin for a relatively long time (>100 ms/cycle).

The ATP-induced myosin head movement was not observed in filaments in which ATPase activity of the myosin heads was eliminated. Application of ADP produced no appreciable myosin head movement. The results of Sugi *et al*. (Sugi *et al*., 1997) showed that the ATP-induced myosin head movement takes place in the absence of the thin filaments.

The rate-limiting step is the release of products of ATP hydrolysis. The hydrolysis of ATP to ADP and Pi requires Mg^{2+} ions. In addition, muscle contraction has a specific requirement for Ca^{2+} ions. The thin filaments are associated with two major actin-binding proteins: tropomyosin and complexes of troponin (Fig. 9). Mechanism by which Ca^{2+} activates the contraction is well established (Solaro and Rarick, 1998; Zot and Potter, 1987).

Sarcomere length affects the maximum force and Ca^{2+}-sensitivity of force in skeletal and cardiac muscle. The dependence of maximum tetanic tension in skeletal muscle, particularly the decline at long sarcomere lengths, has been used to support the cross-bridge model of muscle contraction (Gordon *et al*., 1966), whereas the decline at short sarcomere lengths (the so-called ascending limb) has been less precisely explained. The ascending limb of the length-tension relationship is of great importance in cardiac muscle because it is the sarcomere length range over which the heart normally operates, giving rise to the Frank-Starling relationship. Of additional importance is the increase in Ca^{2+} sensitivity seen with increasing sarcomere length in both skeletal and cardiac muscle. This effect is greater in cardiac muscle and contributes to its greater length dependency of activation, enhancing the Frank-Starling relationship. The reduction in Ca^{2+} sensitivity with decreasing sarcomere length may be most

easily explained by increased distance between thick and thin filament (lattice spacing). Strongly attached cross-bridges contribute to activation, along with Ca^{2+} binding, and the probability of these attachments at a given [Ca^{2+}] decreases with increasing lattice spacing.

Direct measurements of free [Ca^{2+}] in cardiac cells are needed for an understanding of the regulation of contractility. Cyclic variations of cytosolic Ca^{2+} ions are also called calcium transients. In quiescent ventricular muscle, measurements which passed tests for electrode sealing and cell viability gave a mean free Ca^{2+} concentration of 0.26 μM, during contractures Ca^{2+} transients rising as high as 10 μM (Marban, 1980**).** Refinements in techniques for measuring internal calcium suggest a diastolic level about 10^{-7} M, and the systolic peak values up to 10^{-5} M, depending on the contractile state of the myocardium (Opie, 1998). In the presence of sufficient [Ca^{2+}] myosin can interact with actin, which greatly increases the ability of myosin ATPase to hydrolyze ATP. Muscles that are subjected to long period of ischemia become stiff and noncompliant. This is called the rigor state. There is a close association between its development and marked depletion of tissue stores of ATP. In marked contrast to active tension development the development of rigor state does not require the Ca^{2+} ions (Goldman *et al*., 1984; Opie, 1984).

It has been known for a long time that different types of skeletal muscles have varied contractile properties. According to the classic theory of Barany (Barany, 1967) myosin from the fast-twitch muscle has higher ATPase activity and therefore catalyses a more rapid breakdown of ATP. Myosin from slow-twitch muscles, such as heart has a lower ATPase activity, a slower rate of ATP breakdown and a slower rate of contraction.

2.2. Cytoplasmic Ca^{2+} cycle

Changes in intracellular [Ca^{2+}]$_i$ play a critical role in regulating numerous cellular functions, ranging from muscle contraction to neurotransmitter and hormone secretion to gene transcription. The excitation-contraction coupling mechanism involves cyclic changes of the intracellular concentration of free Ca^{2+} ions in cardiac cells, mitochondria participate actively in this process. For us the most important question is whether the excitation-contraction coupling and changes in cytosolic calcium are involved in the regulation of mitochondrial respiration.

2.2.1. Intracellular structures controlling the cytoplasmic Ca^{2+} levels

Skeletal muscle cell has an extensive and well-organized sarcoplasmic reticulum (SR) network with large terminal cisternae abutting the narrow T-tubule. In contrast, cardiac muscle typically has sparser and less rigidly organized SR system with the much larger diameters of T-tubules (Bers, 2002). In myofibrils the SR is greatly enlarged at the terminal cisternae where it comes into close contact from both sides with the relatively narrow T-tubule forming the triad at Z-line level. In mammalian skeletal muscle, T-tubules and the triads are normally at the A-I band junction (Fawcett and McNutt, 1969). The surface of sarcolemma is physically continuous with the membrane of the T-tubule and as such the two combine to form the permeability barrier between the inside of the cell and the extracellular medium. The SR is an intracellular, membrane-bound compartment and it is not continuous with sarcolemma. The volume of SR varies among the cell types and is more abundant in skeletal muscle (Eisenberg *et al.,* 1974; Eisenberg and Kuda, 1975; Eisenberg and Kuda, 1976), than in mammalian heart (Bossen *et al*., 1978; Forbes and Sperelakis, 1995).

Sarcolemma plays an important role in control of the cytoplasmic Ca^{2+} because it is the site at which Ca^{2+} enters and leaves the cell. Indeed, vesicles, isolated from skeletal muscle T-tubules have very high densities of dihydropyridine receptors (DHPR), which are L-type Ca^{2+}-channels (Jorgensen *et al.*, 1989; Wibo *et al.,* 1991). The junctions of SR with sarcolemma in striated muscle are highly specialized and feature bridging structures that have been called “feet” (Franzini–Armstrong, 1972), similar structures are seen in skeletal and cardiac muscle (Frank, 1990). These junctional feet have been identified as the SR Ca-release channels (ryanodine receptor, RyR) (Inui *et al*., 1987a; 1987b).

2.2.2. Ca^{2+} cycling in cardiac myocyte

The cytoplasmic free Ca^{2+} concentration of muscle and other cells at rest is around 20–50 nM; this is by two orders of magnitude lower than the free Ca^{2+} concentration in the extracellular space (usually millimolar) or in the lumen of SR (0.1–2.0 mM). The large Ca^{2+} gradients across cellular boundaries are established and maintained by powerful Ca^{2+} pumps located in the cell surface membranes and in the SR (MacLennan *et al*., 1997; Guerini and Carafoli, 1999; Philipson and Nicoll, 2000), with contributions by the mitochondria (Rizzuto *et al*., 2000; Duchen, 2000). Main features of the cardiac Ca^{2+} cycle are presented in Fig. 10.

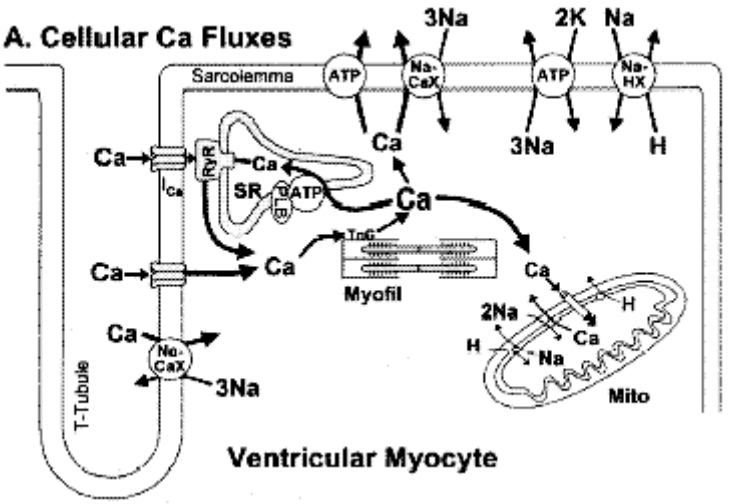


Figure 10. A Schematic diagram of cellular Ca^{2+} fluxes The key pathways involved in myocyte Ca^{2+} transport are shown. During the cardiac action potential L-type Ca^{2+} channels are activated and Ca^{2+} enters the cell via Ca^{2+} current (I_{Ca}) and also a much smaller amount enters via Na/Ca exchange. Ca^{2+} influx controls SR Ca release by the RyR. The Ca^{2+} entry plus the amount released from the SR via CICR raises cytosolic free [Ca^{2+}] causing Ca^{2+} binding to multiple cytosolic Ca^{2+} buffers (Bers, 2000). Ca^{2+} is removed from the myofilaments by the SR Ca-ATPase pump modulated by phospholamban (PLB), sarcolemmal Ca-ATPase pump, Na/Ca exchanger (NCX) and mitochondrial (Mito) uniporter (From Bers, 2000, with permission).

It shows that transsarcolemmal Ca^{2+} influx and SR Ca^{2+} release play major roles in the rise of [Ca^{2+}], which activates contraction in the heart. In skeletal muscle depolarization of the plasma membrane is initiated by the nerve impulse (Sperelakis and Gonzales-Serratos, 2001) and conducted into the interior of the muscle fiber by extensions of T-tubules (Martonosi and Pikula, 2003). The DHPR of the T-tubules and the sarcolemma both serves as a voltage sensor in skeletal muscle and as a Ca^{2+} channel in cardiac muscle by responding to changes in the membrane potential (Hofmann *et al*., 1998; Bezanilla, 2000; Catterall, 2000). DHPR mainly serves as a voltage sensor that controls opening of the RyR channel either by direct interaction with RyR or through contacts with other proteins. The voltage-dependent Ca^{2+} current through the activated cardiac DHPR Ca-channel is fast and sufficiently large to play a fundamental role in the activation of RyR in cardiac SR by a process called Ca^{2+}-induced Ca^{2+} release (CICR) (Cannell *et al.*, 1995; Bers, 2002). CICR was first described in skinned skeletal muscle fibers (Endo *et al.*, 1970; Ford and Podolsky, 1970). Fabiato and Fabiato extensively characterized CICR in series of studies in mechanically skinned cardiac myocytes (Fabiato and Fabiato, 1975

a; b; Fabiato, 1985 a-c; Fabiato, 1992). The myofilaments are activated by the combination of Ca^{2+} influx and CICR and the degree of contractile activation depends how much Ca^{2+} is delivered by this processes to the myofilaments.

For relaxation the Ca^{2+} must removed from the cytoplasm. There are four transport processes that may be involved in this process. Ca^{2+} can be transported: 1) into the SR by the ATP dependent Ca^{2+} pump 2) out of the cell by the sarcolemmal Ca^{2+} pump 3) out of the cell by NCX or 4) into mitochondria via mitochondrial Ca^{2+} uniporter. All these Ca^{2+} transport systems are in direct competition for cytoplasmic Ca^{2+}. For example in rabbit ventricular myocytes, the SR ATP–dependent Ca^{2+} pump removes 70% of the activator Ca^{2+} from the cytosol, and the NCX removes 28%, only nearly 1% leaving each for the sarcolemmal ATPase and mitochondrial Ca^{2+} uniporter (the latter systems collectively referred to as the slow systems).

Apart from the membrane structures, the cardiac and muscle cells possess different proteins for buffering Ca^{2+}. The regulation of Ca^{2+} homeostasis involves a large array of Ca^{2+} binding proteins with a broad spectrum of Ca^{2+} affinities, located in the cytoplasm (Kawasaki and Kretsinger, 1994; Niki *et al*., 1996), in the lumen of SR (Chevet *et al*., 1999; Corbett and Michalak, 2000; Tupling *et al*., 2002), in the nuclei (Bootman *et al*., 2001), and in the mitochondria (McCormack and Denton, 1979; Duchen, 2000; Rizzuto *et al*., 2000; Territo *et al*., 2001). Some of these proteins are Ca^{2+}-sensitive enzymes such as the phosphorylase kinase and the mitochondrial dehydrogenases that adjust the production of ATP to physiological demands of the cell. Others, like calmodulin or Tn serve as Ca^{2+}-dependent regulators of the activity of enzymes such as the plasma membrane Ca^{2+} pump (Martinosi and Pikula, 2003), or actomyosin (Farah and Reinach, 1995). The main amount of Ca^{2+} stored in the SR is bound to the highly acidic protein calsequestrin (Milner *et al*., 1992). Each molecule of calsequestrin binds ~900 nmol Ca^{2+}/mg protein (Bers, 2002). Other Ca-binding proteins in SR include: SR glycoprotein sarcalumenin (Leberer, *et al*., 1989 a; b; 1990), calreticulin (Fliegel *et al*., 1989) and a minor protein — histidine-rich Ca-binding protein (Hofmann *et al*., 1989). From a quantitative standpoint calsequestrin is overwhelmingly dominant compared to the other proteins.

2.2.2.1. Ryanodine receptors

The RyR channel is a poorly selective Ca-channel with very high conductance. Ca^{2+}, Mg^{2+}, ATP, and caffeine regulate the channel. The RyR proteins are present in a variety of tissues, but the highest densities are in striated muscles (Sutko and Airey, 1996; Ogawa, 1994; Olivares *et al*., 1991). RyRs participate in the mechanism of excitation-contraction coupling in striated muscles, but they are also expressed in many other cell types. Three genetically distinct isoforms of RyR exist in mammals (Franzini-Armstrong and Protasi, 1997).

RyR1 is the dominant isoform in skeletal muscle, RyR2 in cardiac muscle, while RyR3 (Fig. 11) is ubiquitously distributed in various tissues including muscle (Ogawa *et al*., 2000). RyR1 or RyR2 knockout mice die early during embryonic development (Takeshima, *et al*.**,** 1989; Takeshima *et al*., 1995). In contrast RyR3 knockout mice lead relatively normal lives and have quite normal striated muscles (Clancy *et al*., 1999, Takeshima *et al*., 1989). Thus, it appears that the physiological role of the RyR3 channel in mammals, unlike that of the RyR1 and RyR2 channels, can be readily compensated for both during development and in the adult. Actually, in several cells two or three RyR isoforms can be co-expressed and interactive feedbacks among them may be important for generation of intracellular Ca^{2+} signals and regulation of specific cellular functions.

The gating of the RyR exhibits a bell shaped dependency on cytosolic Ca^{2+} with activation in the nanomolar to micromolar range and inhibition by millimolar [Ca^{2+}] contsentration (Csordas *et al.*, 2001).

In cardiac myocytes, type 2 RyRs (RyR2s) are activated during excitation-contraction coupling by CICR triggered by Ca^{2+} influx across the sarcolemma. If RyR2s act independently, then the activation and inactivation of individual channels should be stochastic, based on the probability of an individual channel being open or closed. There is about one DHPR for every 5–10 RyR2 channels (Protasi, 2002; Sun *et al*., 1995), and the DHPR and RyR2 channels in heart are not aligned in such a highly ordered fashion as in skeletal muscle (Protasi, 2002; Sun *et al*., 1995). In skeletal muscle, the DHPR and RyR1 are thought to communicate via physical protein-protein linkage (Prozena *et al*., 2002; Protasi, 2002). The membranes of the T-tubule and SR are juxtaposed and separated by a small 10 nm gap. The cytosolic domain of the RyR1 channel spans this narrow gap (Block *et al*., 1986; 1988; Franzini-Armstrong, 1987). Electron microscopy studies show that the skeletal DHPR in the T-tubules are arranged in clusters of four (tetrads). Tetrads represent the structural DHPR/RyR link that allows Ca^{2+} independent coupling in skeletal muscle (Protazi, 2002).

The global Ca^{2+} release during excitation-contraction coupling through RyR appears to be a result of the summation of microscopic Ca^{2+} release events called Ca^{2+} sparks (Cheng *et al*., 1993; Cannell *et al*., 1994; Lopez-Lopez *et al*., 1995). Although it is becoming increasingly clear that Ca^{2+} sparks represent elementary release events of exitation-contaction coupling, their functional organization, in particular whether they are due to activation of a single ryanodine receptor or the concerted opening of many RyRs, remains controversial. Ultrastructural evidence reveals that 22 release channels are present in a typical SR junctional cleft (Franzini-Armstrong and Protasi, 1997). The frequency of Ca^{2+} sparks certainly increases with increasing SR Ca^{2+} load and at certain point macrosparks (more than one locus firing together) and at Ca^{2+} waves is screen (Cheng *et al*., 1996; Satoh *et al*., 1997). It also seems that the Ca^{2+} spark frequency declines at lower SR Ca^{2+} load at unaltered $[Ca]_i$ (Satoh *et al*., 1997) and this is consistent with a continuous modulation of RyR

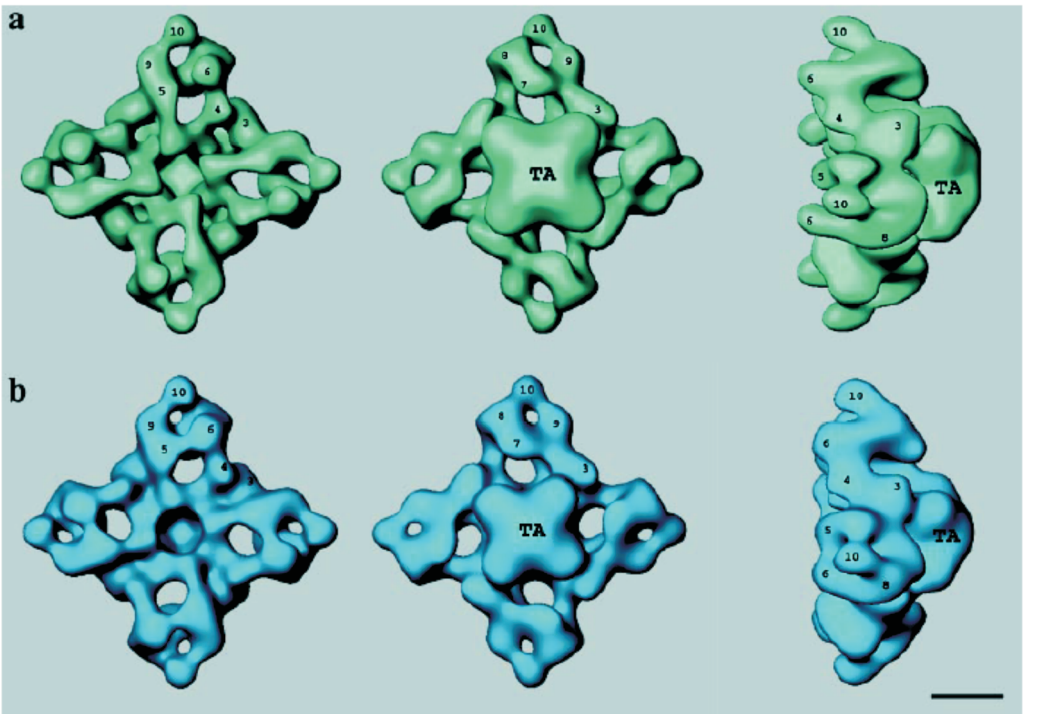


Figure 11. Surface representations of the three-dimensional reconstruction of RyR3 (a) Open state shown in green color and (b) closed state are shown in blue color. The three-dimensional reconstructions are shown for three different views of the receptor, the surface that interacts with the transverse-tubule i.e. the cytoplasmic view (left panels), the face interacting with junctional face membrane of SR, the junctional face (middle panels), and a side view showing the interaction of transmembrane assembly with the cytoplasmic assembly (right panels). The numerals on the receptor indicate the domains as numbered previously by Rademacher and his colleagues (Radermacher *et al*., 1994). Scale bar, 100 Å. (From Sharma *et al*., 2000a, with permission).

gating by $[Ca^{2+}]_{SR}$. However, at low $[Ca^{2+}]_{SR}$ the amplitude of Ca sparks decreases, and the detection of them is difficult (Song *et al*., 1997). In heart Ca^{2+} sparks may be fundamental events underlying both excitation-contraction coupling and resting Ca^{2+} leak from the sarcoplasmic reticulum.

2.2.2.2. Sarcoplasmic reticulum Ca-pump

Kielley and Meyerhoff (1948) were first to describe an Mg-activated ATPase in microsomal fraction from muscle. Ebashi, Hasselbach and Makinose later identified this protein as the membrane associated CaATPase or "relaxing factor" in muscle responsible for lowering cytoplasmic $[Ca^{2+}]$ (Ebashi, 1961; Ebashi and Lippmann 1962; Hasselbach and Makinose, 1961). The SR Ca-ATPase (SERCA) is a member of the P-type ion transporting ATPase family. These pump have high affinity for Ca^{2+} (Km about 0.1 μM), and are capable to maintain a resting cytoplasmic $[Ca^{2+}]$ of 10–20 nM (Martonosi and Pikula, 2003). Like in other P-type ATPases, transient phosphorylation of the enzyme by ATP on an active site aspartyl group is a key step in Ca^{2+} translocation. The density of Ca^{2+} transport sites in the SR membrane is very high (about 30000/μm^2 surface area) and in fast-twitch fibers the Ca^{2+} transport ATPase may account for 80% of the protein content of the SR membrane (Martinosi and Pikula, 2003).

The SERCA family of Ca-ATPases includes 3 major isoforms coded by distinct genes. Two striated muscle SERCA proteins have been sequenced and cloned: they exhibit 84% amino acid identity (MacLennan *et al*., 1985; Brandl *et al*., 1986). Two of these proteins exist in fast-twitch skeletal muscle (SERCA1) and the another in slow-twitch skeletal and cardiac muscle (SERCA 2a), which has four less amino acids compared to SERCA1(Brandl *et al*.,1986, 1987; MacLennan *et al*., 1997). In fast twitch skeletal muscle the SERCA1a isoform predominates in adult, whereas SERCA 1b is present in fetal and neonatal stages. SERCA2b and the third isoform (SERCA3) are ubiquitous in the SR of nonmuscle cells (Dode *et al*., 1996).

Two Ca^{2+} ions are transported per one ATP molecule consumed in both cardiac and skeletal SR (Tada *et al*., 1982; Reddy *et al*., 1996). The Ca^{2+} transport is reversible and under favorable conditions results in the formation of 1 ATP molecule for 2 Ca^{2+} ions released from the lumen of SR (Sumbilla *et al*., 2002). The maximum turnover of cardiac pump has been estimated to be 10–15 Ca ions/pump/sec in dog and quinea-pig (Shigekawa *et al*., 1976; Levitsky *et al*., 1981). This is similar to the turnover rate of the skeletal muscle SR Ca-pump. A major difference between the cardiac and skeletal muscle Ca-pumps is that cardiac muscle contains the phospholamban (PLB) (Tada and Katz, 1982; Tada *et al*., 1998; Simmermann and Jones, 1998). PLB is an endogenous inhibitor in its nonphosphorylated state and inhibits both SERCA1a and SERCA2a, but not SERCA3 (Toyofuku *et al*., 1993; Hikcs *et al*., 1979; Inui *et*

al., 1986). However, PLB can be phosphorylated by cAMP-dependent protein kinase at Ser16 (Tada *et al*., 1974; Simmermann *et al*., 1986). This largely reverses the PLB-induced Ca affinity shift, by increasing the SR Ca-pump activity by 2–3 fold, by changing Km from 300 μM to 100 nM (Bers, 2002). PLB also phoshorylated by CaMkinase II at Thr-17 (Simmerman *et al*., 1986), which produces similar lowering of K_m for Ca^{2+} as phosphorylation of Ser16 (Sasaki *et al*., 1992; Odermatt *et al*., 1996).

Ca^{2+}, pH, ATP, and Mg^{2+} are main regulators for SERCA activity. Generally [ATP] is in excess and $[Ca]_i$ is the limiting substrate for SERCA. When cellular ATP levels fall during ishemia, there may be some decline in SR Ca-pumping and slowing of the muscle relaxation. The actual substrate for the SERCA is MgATP, but other nucleotides can also be used (Tada *et al*., 1978). The pH optimum for the cardiac SERCA is around pH 8, which is more alkaline than that observed in skeletal muscles (Shigekawa *et al*., 1976). Thus acidosis associated with ishemia may be expected to depress the rate of SR Ca pumping.

Since there are many modulators of SERCA, its function can be inhibited by many different agents. The potent inhibitors are thapsigargin (TG) (Thastrup *et al*., 1990), Sagara *et al*., 1992), cyclopiazonic acid (CPA) (Goeger *et al*., 1988; Seidler *et al*., 1989) and 2,5-di (tert-butyl)-1,4-benzohydroquinone (TBQ) (Nakamura *et al*., 1992). TG is of the highest affinity and most selective of these tools. The Ca^{2+} transport activity of SR vesicles purified from skeletal muscle is inhibited by extremely low concentrations of TG (Kd<2 pM) (Davidson and Varhol, 1995). CPA and TBQ can be used as reversible SERCA inhibitors. CPA has submicromolar affinity and ~10μM is typically used to inhibit SERCA. TBQ inhibits the SERCA with a Km~1.5 μM (Nakamura *et al*., 1992) and 10 μM is typically used experimentally.

2.3. Ca^{2+} and mitochondria

The fact that mitochondria may accumulate Ca^{2+} ions was established in the 1960s (Rossi *et al*., 1967; Dhalla, 1969; Harris and Berent, 1969) followed by studies in the 1970s when the ways of mitochondrial extrusion of Ca^{2+} are identified (Fiskum and Lehninger, 1979; Crompton *et al*., 1977). Denton and his colleagues discovered that the three rate limiting enzymes of the citric acid cycle (pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and NAD^+–isocitrate dehydrogenase) are all activated by Ca^{2+} (Denton *et al*., 1972; Denton *et al*., 1978; McCormac and Denton, 1979) suggesting that the rate of ATP synthesis may be influenced by Ca^{2+} uptake. Several groups showed that a physiological increase in cytosolic Ca^{2+} caused an increase in the mitochondrial NADH/NAD^+ ratio (Duchen, 1992). Around the same time, the question, whether mitochondrial Ca^{2+} uptake affects overall Ca^{2+} signaling was investigated (Meyer *et al*., 1988)**.**

Mitochondria may take up vast quantities of free Ca^{2+} when exposed to $[Ca^{2+}]$ of 0.5–1mM (Nicholls and Crompton, 1980). However accumulating evidence, using the fluorescent indicators for cytoplasmic $[Ca^{2+}]$, suggested that $[Ca^{2+}]_c$ rarely reached such high concentrations. It seemed that mitochondrial Ca^{2+} uptake could play only a secondary role and was thus thought to be involved only in "pathophysiologically" high changes in $[Ca^{2+}]_c$. However, more recent investigations showed that $[Ca^{2+}]_c$ signals may not be homogenously distributed in cells and include microdomains of high $[Ca^{2+}]$ (Rizzuto *et al*., 1993; Rizzuto *et al*., 1992). Development of the methods that enabled to directly determine mitochondrial Ca^{2+} signals firmly established that physiological Ca^{2+} signals are transmitted to the mitochondrial matrix in all cells examined thus far.

2.3.1. Mechanisms of mitochondrial Ca^{2+} uptake and release

When mitochondria are energized (i.e. are respiring and translocating protons) Ca^{2+} will move down its electrochemical gradient (about–180 mV) through the inner mitochondrial membrane, into the mitochondrial matrix (Bers, 2002). If mitochondrial electron transport is compromised, and the membrane potential depolarizes to any extent, mitochondrial Ca^{2+} uptake is similarly attenuated.

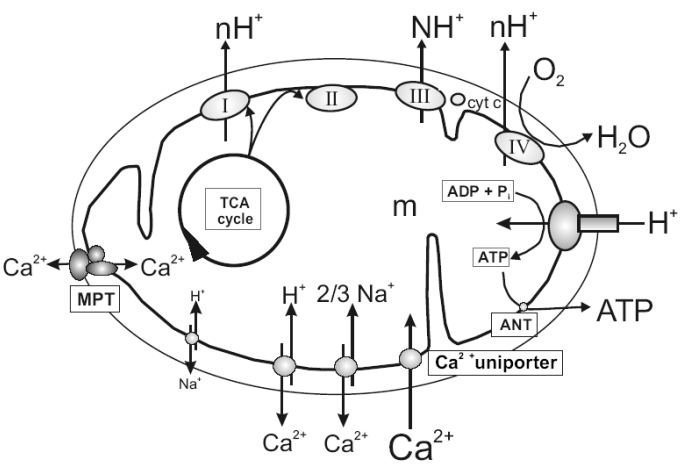


Figure 12. Pathways for Ca^{2+} transport in mitochondria. Cartoon of simplified mitochondria with the main elements of the electron transport chain and proposed ways of Ca^{2+} influx and efflux. The $\Delta\psi m$ provides the driving force for ADP phosphorylation by the F_1F_0 ATPase. Electrophoretic uptake of Ca^{2+} via the uniporter is dependent upon $\Delta\psi m$, and dissipation of the potential abolishes mitochondrial Ca^{2+} uptake. Ca^{2+} is extruded by a Ca^{2+}/Na^+ antiporter. The mitochondrial permeability transition pore (MTP) is a large channel consisting of several mitochondrial proteins. (From Jacobson and Duchen, 2004, with permission)

Ca^{2+} enters via a uniport system which facilitates ion diffusion in the direction of its electrochemical gradient (Fig. 12) without the help of any other source of energy. The Ca^{2+} uniporter is a mitochondrial channel that transports Ca^{2+} and Sr^{2+} but not Mg^{2+}. The uniporter is competitively blocked by physiological concentrations of Mg^{2+} (Nicholls and Ackeman, 1982). The molecular nature of the Ca^{2+} uniporter is not known (the alternative configuration for the pore may be responsible for the 'rapid uptake mode' (RaM)).In respiring mitochondria the kinetics of Ca^{2+} uptake becomes rapidly limited by the rate of H^+ pumping as $[Ca^{2+}]_c$ is raised above about 10μM concentration. The Ca^{2+} uniporter is regulated by a number of modulators (inhibitors and activators). Ruthenium compounds, typically ruthenium red (RR) which is also known to inhibit L-type sarcolemmal Ca^{2+} channels and ryanodine sensitive Ca^{2+} channels, represent a class of noncompetitive inhibitors. The electrophoretic Ca^{2+} uniport is inhibited by RR that also blocks the activation of Ca^{2+}-sensitive dehydrogenases and the Ca^{2+} agonist-induced stimulation of ATP synthesis (Rizzuto *et al*., 2000; Territo *et al*., 2001). A second class of inhibitors is divalent cations that are themselves transported by the uniporter (e.g. Sr^{2+}, Mn^{2+} Fe^{2+}, Ba^{2+} and lanthanides, in descending order of selectivity) (Vainio *et al*., 1970). Inhibition is generally competitive, but not all of the effects are exerted at the transport site(s) because the uniporter is regulated by metal ion binding sites that modulate the affinity for Ca^{2+}. The uniporter is also gated by local adenine nucleotide concentrations (Litsky and Pfeiffer, 1997) in an order ATP>ADP>AMP at a site located at the outer surface of the inner mitochondrial membrane.

Mitochondrial Ca^{2+}uptake also plays an intimate role in the fundamental events involved in cellular $[Ca^{2+}]$ signaling (Duchen, 2000). The equilibration of mitochondrial Ca^{2+} under the influence of the mitochondrial membrane potential is opposed by Na^+-dependent and Na^+-independent Ca^{2+} exporters (Bernardi, 1999; Rizzuto *et al*., 2000), and by a non-selective high-conductance PTP (Rizzuto *et al*., 2000) that facilitate the export of Ca^{2+} from the mitochondrial matrix space back into the cytoplasm.

The Na^+-dependent Ca^{2+} efflux is mediated by the mitochondrial NCX exchanger and is primarily responsible for balancing the electrophoretic Ca^{2+} uniport. The mitochondrial NCX operates with a probable stoichiometry of 2 or 3 Na^+/1 Ca^{2+}, its Km for Na^+ is 8–10 mM and its Vmax is 18 nmol Ca^{2+}/mg protein per min in the heart (Martonosi and Pikula, 2003). Originally thought to exchange two Na^+ for one Ca^{2+}, the exchanger was thus believed to be passive and electroneutral (Brand, 1985; Wingrove and Gunter, 1986). However, the observation that mitochondria could extrude Ca^{2+} uphill against a Ca^{2+} gradient energetically twice that of the Na^+ gradient (Brierley *et al*., 1994; Baysal *et al*., 1991) suggests that the ion exchange cannot be electroneutral, and a stoichiometry of $3Na^+/2Ca^{2+}$ has been suggested. The Na^+-independent Ca^{2+} efflux is insensitive to RR and may occur *via* an nH^+/Ca^{2+} exchange mechanism (Kunz, 2001) identified in various tissues, including brain, heart and liver.

2.3.1.1. Ca^{2+} rapid uptake mode

An alternative route of mitochondrial Ca^{2+} uptake has also been described, the rapid uptake mode, or RaM (Sparagna *et al*., 1995). Liver and heart mitochondria contain a RR-insensitive (the inhibition requires much higher concentrations) Ca^{2+} uptake mechanism, designated as RaM. (Bernardi, 1999; Rizzuto *et al*., 2000; Buntinas *et al*., 2001). The RaM has much higher conductivity at the beginning of a pulse than the uniporter, but this conductivity rapidly declines as the cytoplasmic [Ca^{2+}] rises due to Ca^{2+} binding to an external regulatory site (Buntinas *et al.*, 2001).

When isolated liver mitochondria were exposed to short pulses of labeled Ca^{2+}, it seemed that mitochondria were capable of very high rates of Ca^{2+} uptake, which were quickly inhibited, presumably by the rising of intramitochondrial [Ca^{2+}]. This ability to rapidly absorb very short transients of Ca^{2+} has been generally observed in all tissues **(**Gunter *et al*., 2000). Investigation by Butinas *et al*. (Butinas *et al*., 2001) showed that RaM functions in cardiac mitochondria with some of the characteristics of RaM in liver, but its activation and inhibition are quite different. It is feasible that these differences represent different physiological adaptations in these two tissues. In both tissues, RaM is highly conductive at the beginning of a Ca^{2+} pulse, but is self-inhibited by the rising [Ca^{2+}] of the pulse itself. In cardiac mitochondria (cardiac and brain mitochondria seemed to behave similarly (Sparagna, 1995)), the time required at low [Ca^{2+}] to reestablish high [Ca^{2+}] conductivity via RaM i.e. the "resetting time" of RaM is much longer than in liver. RaM in liver mitochondria is strongly activated by spermine, activated by ATP or GTP and unaffected by ADP and AMP. In heart, RaM is activated much less strongly by spermine and unaffected by ATP or GTP. RaM in heart is strongly inhibited by AMP and has a biphasic response to ADP; it is activated at low concentrations and inhibited at high ADP concentrations (Buntinas *et al*., 2001).

2.3.2. Mitochondrial permeability transition

Mitochondria contain a structure that could form a large unspecific pore under conditions of high matrix [Ca^{2+}] (Al Nasser and Crompton, 1986), Pi concentrations and oxidative stress (Harworth and Hunter, 1980). When the pore opens, solutes up to about 1.5 kDa can pass through the inner membrane (Hunter *et al*. 1976), a process known as the mitochondrial permeability transition. Subsequently, the membrane potential decays, oxidative phosphorylation uncouples from electron flow, intramitochondrial ions and metabolites are released, and a large amplitude swelling can occur, disrupting the outer membrane and releasing intermembrane compounds. The exact molecular composition of the PTP is not known. Some researchers have linked ANT and VDAC to the PTP formation that results in the release of Ca^{2+} ions and

metabolites from the matrix space (Szabo and Zoratti, 1993; Szabo *et al*., 1993, Halestrap *et al*., 2002). One view is that combining VDAC with a pore through the inner membrane results in a pathway for the transfer of solutes through both membranes. Another hypothesis (Marzo *et al*., 1998) proposes that the PTP formation results in osmotic swelling of the matrix compartment and consequently sufficient physical stress is applied to the outer membrane, culminating in rupture of that membrane. Since the protein concentration in the matrix is higher than in the intermembrane space or cytosole, water reaches the matrix causing the swelling and the outer membrane is broken because its area is much smaller that of the inner membrane and all the intermembrane proteins are released to the cytoplasm (Skulachev, 1996; 2000).

Other authors described PTP as the multiprotein complex at the inner/outer membrane contact sites of mitochondria (Fig. 13). Molecules involved may include the peripheral benzodiazepine receptor from the outer membrane, VDAC, hexokinase and CK, ANT, and cyclophilin D (Zoratti and Szabo, 1995; Halestrap and Davidson, 1990). The participation of complex I has also been suggested (Fontaine and Bernardi, 1999).

The PTP is voltage-dependent, remaining closed at high membrane potentials and opening with depolarization (Bernardi, 1992). The PTP could be specifically blocked by cyclosporin A, which binds to CyP-D. (Crompton and Costi, 1988; Halestrap and Davidson, 1990). Adenine nucleotides are also strong inhibitors of PTP opening, with ADP producing a stronger effect than ATP or AMP (Zoratti and Szabo, 1995). Strong support for the central role of ANT in the PTP is provided by the single-channel current measurements in patch-clamp experiments with the isolated reconstituted ANT (Brustovetsky *et al*., 2002). This probably involves the ANT, and the direction it faces. ANT inhibitors such as atractyloside and BKA enhance or reduce the probability of PTP opening (Schultheiss and Klingenberg, 1984). Two questions are still discussed: firstly, whether the ANT is the only structure that causes permeability transition and secondly, whether the ANT forms the pore only as a complex with VDAC (Vyssokih and Brdizska, 2004)**.** PTP opening is also inhibited at low mitochondrial matrix pH (protonation of His residues keeps the channel closed).

When a "lawn" of isolated mitochondria is plated onto a coverslip and watched on by epifluorescence microscope, individual mitochondria may be seen to undergo repeated depolarizations, followed by near-complete repolarizations. The flickering depolarization was inhibited by cyclosporin A (Huser *et al*, 1998). Several groups have suggested that flickering, transient openings of the pore may serve a physiological role as a mechanism to remove calcium rapidly from mitochondria (Altschuld *et al*., 1992). Ichas and Mazat have proposed a biophysical model in which flickering of PTP may play an active role in Ca^{2+} signaling and initiation of Ca^{2+}-dependent apoptosis) (Ichas and Mazat, 1998).

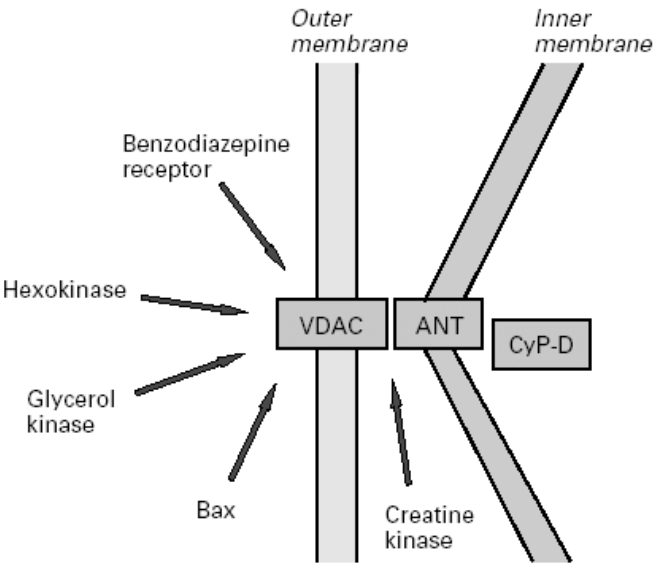


Figure 13. PTP pore topology. The basic unit of the PTP is the VDAC-ANT-CyP-D complex located at contact sites between the mitochondrial inner and outer membranes. The likely PTP structure incorporates the complex formed by apposition of VDAC and ANT at contact sites between the mitochondrial outer and inner membranes together with matrix CyP-D. The association between kinases (e.g. hexokinase, glycerol kinase) and VDAC/ANT is believed to provide a conduit whereby ATP generated by OxPhos is channeled directly to the kinases. ATP utilized by mitochondrialy bound hexokinase is derived mainly from oxidative phosphorylation rather than from the cytosol (McCabe, 1994). The properties of the kinase-enlarged complex add further support to the basic VDAC/ANT/CyP-D model. Thus Brdiczka and co-workers have reconstituted pore activity in planar bilayers and in liposomes from preparations that contain VDAC, ANT and CyP-D along with a number of other proteins (Beutner *et al*., 1996; Beutner *et al*., 1998; Halestrap *et al*., 1997). (From Crompton, 1999, with permission)

The formation of PTP leads to a number of deleterious effects for cell survival. Among these is the release of soluble factors (including cytochrome c) that may activate caspases (Cai *et al*., 1998). The release of cytochrome c into the cytoplasm may be necessary for the initiation of apoptosis (Yang *et al*., 1997), and it has been suggested that PTP formation is the rate-limiting event of apoptosis (Hirsch *et al*., 1997). Interestingly, some evidence suggests that in the presence of ATP (necessary for apoptosis) the PTP leads to apoptosis, while in the absence of ATP the PTP leads to necrosis (Qian *et al*., 1999; Richter *et al*., 1996). ATP is required for the development of apoptosis. Consistent with the ATP dependence of caspase 9 activation, cellular apoptosis in many systems requires ATP (Leist *et el*., 1997; Eguchi *et al*., 1997; Richter *et al*., 1996). When ATP is depleted, apoptosis is blocked.

3. Energy transfer by creatine kinase system and its regulation

3.1 Creatine kinases

CKs are guanidino kinases that catalyze the reversible transfer of a high-energy phosphate moiety between ATP and creatine (Cr), generating ADP and the high-energy phosphate carrier, phosphocreatine (PCr). The reaction was discovered by Karl Lohmann (Lohmann, 1934) as noted above:

$$PCr + MgADP + H^+ \rightleftarrows Cr + MgATP \quad (2)$$

The CK reaction plays a central role in the intracellular energy transfer. CK isoenzymes, specifically located at places of production and utilization of ATP, are linked by a PCr/Cr circuit and found in cells with intermittently high — energy demands. Family of CK is encoded by four independent genes, each coding different monomeric isoforms. Isoforms of CK are classically divided into two groups−mitochondrial (MitCK) and cytosolic isoforms (cytosolic muscle (MCK) and brain (BCK) isoforms). In cytosole CK is present in dimeric form. Expression of the cytosolic CK`s is highly regulated during development: BCK predominates in embryonic tissues and drops in the postnatal period thereby creating an isoenzyme switch with MCK (Trask and Billadello, 1990). MCK is markedly upregulated in sarcomeric tissues after birth (Payne and Strauss, 1994). In mammals and birds the two tissue-specific "cytosolic" CK isoforms can be distinguished from a ubiquitous BB-CK form, expressed at high levels in brain, and a muscle-specific form, MM-CK expressed at high levels in striated muscles (Eppenberger *et al*., 1964; Eppenberger *et al*., 1967). Four MM-CK-specific and highly conserved lysine residues are thought to be responsible for the interaction of MM-CK with the M-band (Brdiczka and Wallimann, 1994).

The total activity of CK isoforms decreases in the following order: fast-twitch skeletal muscle, slow-twitch skeletal muscle, cardiac muscle, but the activity of MitCK increases in the same order and its activity reaches 30–40% of total enzyme activity in hearth (Saks *et al*., 1974; Iyengar, 1984; Yamashita, 1991). The coupled CK isoenzymes function in steady state via a mechanism of functional coupling (metabolic channeling), but in different directions depending on their localization.

3.2. The phenomenon of functional coupling

3.2.1. Functional coupling between mitochondrial creatine kinase and adenine nucleotide translocase

MitCK is localized within the mitochondrial intermembrane space (Wyss *et al*., 1992; Scholte, 1973a; b) and are known to bind strongly to the outer surface of the inner mitochondrial membrane (Saks *et al*. 1994). In contrast to the cytosolic isoforms, MitCKs can occur as dimers and octamers, the latter being built up by association of two dimers . Octameric MitCK can form stable cross-links between the inner and outer membranes thereby inducing membrane contact sites *in vitro* (Rojo *et al*., 1991). Whereas the cytosolic CK isoforms mainly use PCr to reproduce ATP at sites of energy consumption, such as the myofibrillar actomyosin ATPase in muscle (Wallimann *et al*., 1984; Ventura-Clapier *et al*., 1994) and the Ca^{2+}-ATPase of the SR (Rossi *et al*., 1990; Minajeva *et al*., 1996), the mitochondrial isoform is mainly responsible for the turnover of ATP, which is exported out of the mitochondrial matrix by ANT, into PCr (Klingenberg, 1993). Mitochondrial isoform called "ubiquitous" (uMitCK) is expressed in intestine, brain, kidney, placenta and in very low level in aorta and sarcomeric tissues. The other MitCK isoform is sarcomeric MitCK (sMitCK), which is exclusively expressed in heart and skeletal muscle.

The coupled ANT-MitCK system is an excellent example of the coupling in supramolecular complexes of enzymes and transporters, which can be described by following formulae (Saks *et al.*, 2004):

Functional coupling = Metabolic channeling + Microcompartmentation (3)

In mitochondria, the MitCK is bound to the external surface of the inner membrane by cardiolipin in close association to the ANT (Saks *et al*., 1975, Jacobus and Lehninger, 1973, Jacobus, 1985, Muller *et al*., 1985; Barbour *et al*., 1984). Cardiolipin is situated in mitochondrial inner membrane, binds ANT and is considered to serve as contact site for MitCK (Fig. 14). Three C-terminal lysines determine the high affinity sMitCK/cardiolipin interaction and its effects on MitCK structure, while low affinity binding and some effect on membrane fluidity depend on other structural components (Schlattner *et al*., 2004).The architecture of the complexes of these proteins may be different in different locations: dimer to dimer, or octamer of MitCK in complex with a cluster of ANT (Walliman *et al*., 1992). The Wallimann group and several other authors favor the point of view that the normal state of the MitCK in cardiac mitochondria is an octameric form (Walliman *et al*., 1992, Wyss and Kaddurah-Daouk, 2000). Direct linkage of octameric MitCK to tetrameric form of ANT involving as cardiolipin was initially proposed by Schnyder and Wallimann (Schnyder *et al*., 1994), but the MitCK: ANT monomer stoichiometry proved to be 1:2 (Kuznetsov and Saks, 1986). Strong proof in favor of the 1:2

stoichiometry and for the possibility of structural proximity between MitCK and ANT was the work where MitCK in the mitoplasts were treated with either inhibitory or noninhibitory antibodies against MitCK (Saks *et al*., 1987). Noninhibitory antibodies from rabbit with high affinity to rat mitochondrial CK inhibited neither CK activity nor OxPhos, Inhibitory antibodies succeed to inhibit both MitCK and oxidative phosphorylation. These data show close and specific spatial arrangement of MitCK and ANT in mitochondria.

In isolated cardiac mitochondria the maximal activity of MitCK in direction of PCr production is exactly equal to the maximal rate of the ATP production in the oxidative phosphorylation (close to 1 μmol/mg/min at 30°C) (Saks *et al*., 1975, Jacobus and Saks, 1982). There is no excess capacity of the MitCK, as assumed by Balaban (Balaban, 2002) and Korzeniewski (Korzeniewski and Zolade, 2001) because of very precise localization of MitCK with respect to ANT, the ATP is directly channeled by ANT from matrix into micro-compartment ('gap') between these proteins (Saks *et al*., 1975; Barbour *et al.*, 1984; Jacobus and Saks, 1982; Saks, *et al*, 1985) and quickly saturates the active site of MitCK. The local ratio of ATP/ADP available for MitCK in the gap between ANT and MitCK is always much higher than that in the medium and therefore forces, both thermodynamically and kinetically, the MitCK reaction to proceed in direction of PCr synthesis (Saks *et al*., 1975; Barbour *et al*., 1984; Jacobus and Saks, 1982; Saks, *et al*., 1985). Channeling of ATP and ADP into this microcompartment and their turnover between MitCK, ANT and OxPhos represents the mechanism of functional coupling between these systems and is the basis of metabolic stability of the heart (Saks *et al*., 1975; Jacobus and Saks, 1982; Saks *et al*., 1985; Saks *et al*., 1994). The importance of the functional coupling of MitCK with ANT for metabolic stability of the heart was recently verified by Spindler (Spindler *et al*., 2002) in a study of isolated perfused transgenic hearts with the deficiency of the sarcomeric form of MitCK: in these hearts the PCr/ATP ratio was considerabely decreased. Functional coupling of MitCK and ANT also prevents from PTP opening

An important property of the CK system is that its total activity, its isoform distribution, and the concentration of guanidino substrates are highly variable among species and tissues. It has been shown that in the highly organized structure of adult muscles, specific CK isoenzymes are bound to intracellular compartments, and are functionally coupled to enzymes and transport systems involved in energy production and utilization. The fast-twitch muscle cells are inable to maintain constant PCr levels during continuous burst of tetanic contractions of high intensity and the developing metabolic instability progresses into fatigue. During period of rest the oxygen debt is paid back due to activation of mitochondrial respiration, and the cellular sources of PCr are replenished due to PCr synthesis predominantly via MitCK coupled to ANT, until the high PCr/Cr ratio typical for the resting state is achieved (Saks *et al*., 1977; Walsh *et al*., 2001; Mahler, 1985; Greenhaff, 2001). In the skeletal muscle, like in the myocardium, the MitCK is tightly coupled to ANT

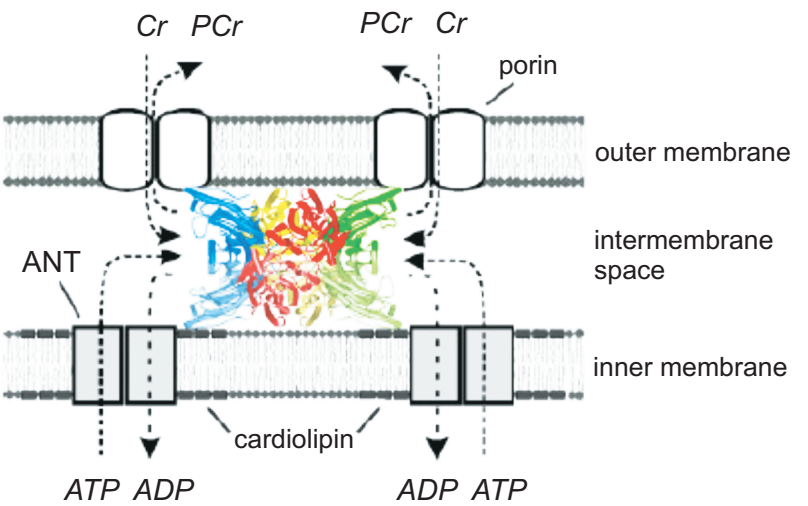


Figure 14. MitCK in mitochondrial contact sites. Model of the topology of MitCK, ANT and mitochondrial porin in the peripheral contact sites, where MitCK cross-links outer and inner mitochondrial membranes. The direct interaction of MitCK with porin and cardiolipin exists, as well as the close vicinity to ANT. Substrate and product pathways are indicated (From Shlattner *et al*., 2004, with permission).

(Kupriyanov *et al*., 1980) and the respiration rate in the skeletal muscle cells is controlled by the CK system and PCr/Cr ratio (Saks *et al*., 1977; Walsh *et al*., 2001; Mahler, 1985).

Gellerich has proposed that the functional coupling can be improved by controlling the permeability of the outer membrane VDAC by changing oncotic pressure (Gellerich *et al.*, 1994). This may be important under *in vivo* conditions.

3.2.2. Functional coupling between MM creatine kinase and MgATPases

The fraction of CK that is bound to the M-band (see 3.1) is functionally coupled to the myofibrillar actin-activated MgATPase (Wallimann *et al*., 1984; Ventura-Clapier, *et al*., 1994). Only the muscle-specific M-type MM-CK, but not the highly homologous B-type BB-CK, is able to interact with this sarcomeric structure (Stolz and Wallimann, 1998). Hornemann (2003) for the first time shows unequivocally a strong interaction of MM-CK with myomesin and with the highly homologous M-protein. Although titin domains display a high degree of similarity with the myomesin and M-protein domains, they did not observe an interaction of MM-CK with purified titin (in the BiaCore analysis). Cytosolic CKs, in close conjunction with Ca-pumps, play a crucial role for the energetics of Ca-homeostasis (Wallimann *et al*., 1998). In 1962 Yagi and Mase showed in a reconstituted system that the Michaelis-Menten constant of the ATPase reaction was 1–2 orders lower when CK was associated with myosin shifting the Km value from 300 to 7 μM, they proposed, that the substrate concentration near the ATPase active site would be much higher than the mean concentration in the reaction mixture (Yagi and Mase, 1962). Saks *et al*. showed that ADP produced by ATPases bound to CK with high affinity (Saks *et al*., 1976). It has been found that a purified heart plasma membrane preparation contains high CK activity. CK isoenzyme bound to plasma membrane of heart cells is identical to MM-CK and is able to rephosphorylate effectively ADP formed in the Na,KATPase reaction. This conclusion is based on observation that the rate of PCr splitting in the plasma membrane preparations is sensitive to ouabain and is determined by the kinetic parameters both of the Na,KATPase and plasma membrane CK (Saks *et al*., 1976). Bessmann and his colleagues showed by using radioactive labeled phosphate that ATP formed from PCr through CK's reaches active site of ATPase much more easily than ATP added externally (Bessmann *et al*., 1980). Using the competetitive pyruvate kinase/phospho-enolpyruvate (PK/PEP) system for ADP produced in CK reaction shows also strong functional coupling between CK and ATPases (Saks *et al*., 1984). In these experiments soluble enzymes were compared to myofibrils bearing both ATPase and CK activities. The production of Cr and pyruvate in the soluble enzyme system depended directly on the activities of both enzymes. In the

myofibrillar preparations, even PK/CK ratio of 100 was not enough to inhibit the production of Cr (Saks *et al*., 1984), showing preferential access of ADP to CK reaction due to immobilization of the two enzymes. The nature of coupling has been carefully morphologically studied (Wegmann *et al*., 1992) and despite that the distance between CK and ATPase is more than 10 nm, i.e. in the situation where the coupling between the enzymes should been lost (Fossel and Hoefeler, 1987), the kinetical data proved the existence of strong coupling (Krause and Jacobus, 1992). Therefore the co-localization on a surface of enzymes which catalyze consecutive reactions, may increase the efficiency of their kinetic coupling. This vicinity may is able to favor the channeling of metabolites. Such channeling has also been observed in Triton-X100 treated frog heart cells: ATP was channeled from CK to the ATPase site of myosin and do not diffuse into the bulk solution (Arrio-Dupont, 1988).

Similarly, MM-CK is strongly bound to SR membranes, in which it is functionally coupled to the CaATPase, and ensures efficient energy provision of the SR by the local regeneration of ATP (Levitskii *et al*., 1977; Rossi *et al*., 1990, Korge *et al*., 1993; 1994; Minajeva *et al*., 1996). ATP regenerated by endogenous CK was not in free equilibrium with the ATP in the surrounding medium but was used preferentially by SR CaATPase for Ca^{2+}-uptake (Korge *et al*., 1993). Efficient translocation of ATP from CK to SR CaATPase, despite the presence of an external ATP trap in the surrounding medium, can be explained by close localization of CK and CaATPase on the SR membranes. Several factors (amount of membrane-bound CK, oxidation of SH groups of CK, decrease in [PCr]) can influence the ability of CK/PCr system to support a low ADP/ATP ratio and fuel the Ca-pump with ATP (Korge *et al*., 1993).

In the cytoplasm the MM-CK reaction is coupled to the glycolytic system, which ensures the use of ATP produced in phosphoglycerate kinase and PK reactions. Interaction between the glycolytic system and OxPhos can be effectively regulated by CK system: high PCr to Cr ratio reached in the cytoplasm due to OxPhosp inhibits the overall glycolytic flux due to limited availability of ADP (Kupryanov *et al*., 1980).

3.3. Metabolic consequences of functional coupling of CK

The system consisting of CK, Cr and PCr, which is related to the ATP-ADP system has two clearly defined functions: the temporary energy buffering which serves the purpose of maintaining an adequate ATP/ADP ratio in the cells with increased energy demand (McGilvery and Murray, 1974; Connett, 1988) and the spatial energy buffering (spatial energy buffering–maintaining local ATP/ADP ratios) or energy transport function (Saks *et al*., 1994; 1998a; 2004; Wallimann and Hemmer, 1994; Ventura-Clapier *et al*., 1994). A practical aspect of temporary buffer function is that it allows one to calculate the free

cytoplasmic ADP concentration in the cells around 50 μM, witch cannot be measured either biochemically or by NMR, and this aspect is widely exploited in practically all modern metabolic studies (Saks *et al*., 1996). This concept is a satisfactory explanation for some events (concentration values, fluxes, etc.), but it is not sufficient t o explain the existence of different CK isoenzymes (that possess very similar kinetic and thermodynamic characteristics and uniform conserved structure of the active center (Muhlebach *et al*., 1994)), as well as the appearance of MitCK during evolution.

The spatial buffering or energy transport function is the shuttling of PCr and Cr between sites of energy utilization and energy production. In this role PCr is thought to function as an "energy carrier" connecting sites of mitochondrial OxPhos with sites of energy utilization (Ventura-Clapier, 2004; Saks *et al*., 2004). Joubert experimentally demonstrated of the role of CK in both the energy buffering and shuttling through CK isoforms in the whole heart and myocardium and evidenced the physiological versatility of the pathways transferring energy by ATP and PCr (Joubert *et al*., 2004).

This function of CK is supported: (i) by the specific subcellular compartmentation of different CK izoenzymes (Wallimann *et al*., 1992), (ii) by evidence indicating subcellular compartmentation of PCr/Cr, ATP/ADP and Pi (Saks *et al*., 1984; 1991; Savabi, 1994; Gellerich *et al*., 1987; Zeleznikar and Goldberg, 1991) (iii) by the localization, structure and functional properties of the octameric MitCK (Wyss and Wallimann, 1992; Wallimann *et al*., 1992; Wyss *et al*., 1992) (iv) by in *vivo* ^{18}O labeling of the phosphryl moieties of metabolites in intact diaphragm muscle showing that discrete adenine nucleotide pools exist in the cells and the rates of appearance of [^{18}O]PCr are consistent with CK-catalyses phosphoryl exchange functioning in an obligatory PCr shuttle (Zeleznikar and Goldberg 1991), and (v) the functional coupling phenomena described in previous section

Analysis of the equilibrium equation showed that in fact about 99% of the energy flux via equilibrium CK is represented by that of PCr via a mechanism of facilitated diffusion (Meyer *et al*., 1984). This also modifies the concept of energy buffer role of CK into that of metabolic capacity system with spatial buffering (spatial energy buffering–maintaining local ATP/ADP ratios) (Meyer *et al*., 1984; Meyer, 1988; Sweeney, 1994). It takes into consideration the structural organization of the cells — that the cytoplasmic space contains a significant amount of MM-CK between mitochondria and myofibrils. A combination of facilitated diffusion (defined as a process of passive transport; via which molecules diffuse across membranes with the help of transport proteins) of high-energy phosphate bonds in the cytoplasm with the energy store function confers on the CK system the property of metabolic capacity (Meyer 1988; Sweeney 1994).

Theories of metabolic control describe the control of energy fluxes quantitatively by flux control coefficients, and one of the basic theorems states that the sum of control coefficients is one in the case of homogeneous system

(Westerhoff and Van Dam, 1987). Kholodenko with his colleagues developed this theory further, showing that in the system of metabolic channeling the sum of flux control coefficients significantly exceeds one (Kholodenko *et al*., 1993 a; b; c; Kholodenko *et al*., 1995) and such systems are controlled more efficiently than simple, homogeneous metabolic capacity system. Fully coupled systems the sum of flux control coefficients may have a value higher than 8 (Saks *et al*., 1996) and this is very different from flux coefficients for homogeneous systems.

Mice missing sMitCK showed no effect on skeletal or cardiac muscle while mice missing MMCK have decreased skeletal muscle performance (Van Deursen *et al*., 1993). No effect on cardiac muscle has been described in the heart, and no contractile phenotype was detected in diaphragm muscle of MM-CK knockout mice (LaBella *et al*., 1998; Saupe *et al*., 1998). In addition, expressing the brain BB-CK isoform in the muscle of mice missing MMCK reversed the contractile defect and metabolic alterations seen in the MM-CK knockout mice. Kaasik *et al*. (2003) proposed that oxidative capacity of fast-twitch muscle was increased twofold in cytosolic and mitochondrial CK isoforms knockout mice. In these mice, mitochondrial ability to provide ATP for Ca^{2+} uptake and relaxation of rigor tension was dramatically enhanced. Deletion of the cytosolic MCK reduced CK-catalyzed phosphotransfer by 20%, while the absence of the mitochondrial sMitCK isoform did not affect PCr metabolic flux (Dzeja *et al*., 2004). The efficiency of stimulation of mito-chondrial respiration in permeabilized muscle cells by ADP produced at different intracellular sites, e.g. cytosolic or mitochondrial intermembrane space, was evaluated in wild-type and CK-deficient mice (Kay *et al*., 2000). Stimulatory effect of Cr, observed in wild-type cardiac fibers disappeared in MitCK deficient, but not in cytosolic CK-deficient muscle. It is concluded that respiration rates can be dissociated from cytosolic [ADP], and ADP generated by mitochondrial CK is an important regulator of OxPhos.

Nevertheless mice lacking one or both of the MM-CK and MitCK isoforms are viable and develop almost normal cardiac and skeletal muscle function under the conditions of moderate workload (Steeghs, 1998), suggesting adaptations or other mechanisms that may ensure phosphotransfer. Crozatier showed the specific role of CK in excitation-contraction coupling in cardiac muscle that cannot be compensated for by other pathways (Crozatier, 2002). While fixed CK is essentially important, other systems could also be involved as well, such as bound glycolytic enzymes or adenylate kinase (Ventura-Clapier *et al*., 2004). Redistribution of phosphotransfer through glycolytic and AK networks contributes to energetic homeostasis in muscles under genetic and metabolic stress complementing loss of CK function (Dzeja *et al*., 2004).

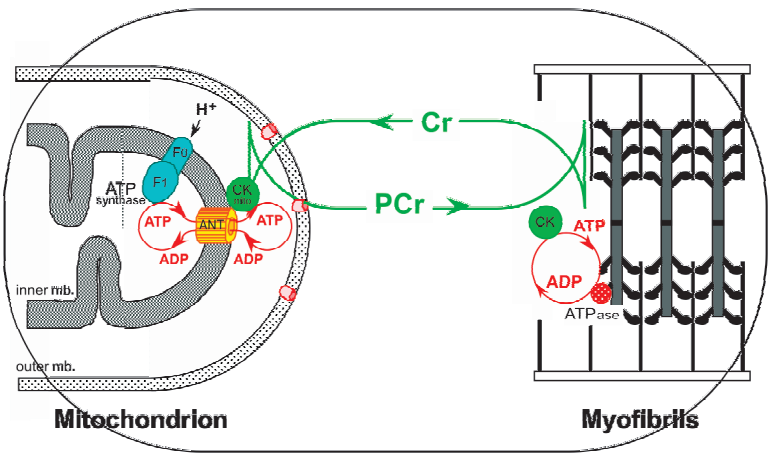


Figure 15. The PCr pathway for intracellular energy transport. The transport of the high energy phosphate achieved by the chain of CK molecules transporting the ATP bound from the site of energy production (mitochondria) to the sites of energy consumption (myosin ATPases, ion transport channels) and transducting the feedback signal back to mitochondria. The transport is carried out over local pools of ATP, ATP, PCr and Cr without changes in the total concentrations of adenine nucleotides. That function of the shuttle consisting of coupled CK molecules is best illustrated by the fact that the cardiac function is totally dissociated from the tissue ATP content–phenomenon difficult to explain by other means (Neely and Grotyohann 1984; Kupriyanov *et al*., 1987, Hoerter *et al*., 1988). (From Saks *et al*., 2000, with permission).

4. Regulation of respiration *in situ*

Much of information regarding the regulation of cellular respiration has been gained from the experiments with isolated mitochondria. The main conclusion is that the need for the ATP in cells is not constant and therefore exists the need to strictly control the production of ATP according to the demand from the energy consuming processes in the cells. One of the main mitochondrial characteristics is the dependence of the oxygen consumption rate on free ADP concentration.

Lardy and Wellmann experimentally demonstrated the existance of respiratory control and the uncoupling effect of the dinitrophenol on respiration of isolated mitochondria (Lardy and Wellman, 1952). Few years later Chance and Williams proposed widely known theory of the regulation of OxPhos through a negative feedback involving ADP (Chance and Williams, 1956). This method where dual-wavelength spectrophotometer was used in combination with an oxygen electrode, allowed to siultaneously determine the states of different electron carriers in the metabolic states.

For convenience, Chance and Williams numbered the various possible conditions of mitochondria: Sate 1 – only mitochondria in medium without ADP and respiratory substrates. State 2 – substrate added, respiration low owing to lack of ADP. State 3 – limited amount of ADP added allowing rapid respiration. State 4 – all ADP converted to ATP, respiration slows. State 5 – anoxia

Isolated mitochondria *in vitro* are considered to be characterized by very high affinity for ADP. For isolated mitochondria *in vitro* Km value was found to be 10–20 μM (Lardy and Wellman, 1952; Chance, 1952). Gellerich *et al*. proposed (Gellerich *et al*., 1998; 2002) that earlier studies were done on isolated mitochondria suspended in isotonic media without macromolecules, in which intermembrane space of mitochondria is enlarged and macromolecules can restore the morphological changes which occur upon isolation of mitochondria in normally used isolation media. It was shown that in the presence of dextrans the permeability of mitochondrial outer membrane for adenine nucleotides decreases, which may have considerable implications for the transport of ADP into the mitochondria. Assuming that the dextran concentration of 15% mimics the intracellular oncotic pressure on mitochondria *in vivo*, the apparent Km (ADP) of OxPhos within the intact cell seems to be about 50 μM which is somewhat higher than the cytoplasmic free ADP concentration as reported for the intact heart (Gellerich *et al*., 1998; 2002).

The method of isolated mitochondria allows to perfectly controlling the medium composition around the organelles, it is reliable, rapid and relatively cheap. However, isolation of mitochondria by homogenization of tissue and sedimentation of mitochondria by centrifugation can seriously affect structural and functional properties of the organelles. Disruption of the cellular architecture is likely to alter the parameters concerning the interaction between

the mitochondria and the extramitochondrial space and to destroy the organized structure of cytoplasm. This is particularly important if the mitochondria are being isolated from tissue already damaged by a pathological process. Furthermore, the limited mitochondrial yield typically (10–20%) achieved during the procedure of isolation does not allow to investigate the total tissue mitochondrial population.

4.1. Regulation of respiration by ADP: skinned fibers

The concept of regulation of respiration by ADP cannot explain the observation that in cardiac cells the respiration rate increases parallel to workload without increase in ADP. This contradiction emphasizes that regulation of respiration should be very different in isolated mitochondria and intact cells. In this regard a qualitatively new information has been obtained from the studies on permeabilized fibers.

The idea to use a detergent for permeabilization of the plasmalemma to study the functioning of intracellular membrane organelles actually came from a work of Endo and Kitazawa (Endo and Kitawa, 1978). The use of specific membrane permeabilization with detergents (saponin, digitonin) (Glauert *et al*., 1962) allows for the study of organelle function while maintaining the cellular architecture and controlling the intracellular milieu (Veksler *et al*., 1987; Saks *et al*., 1991; 1993; 1994; 1995; Kaasik *et al*., 2001). Saponin and digitonin, due to the hydrophobic steroid core, have high affinity for cholesterol and preferentially extract cholesterol from membranes, therefore they specifically "attack" cholesterol-rich membranes such as the plasmalemma (Korn, 1969). Endoplasmic reticulum has much lower cholesterol content; inner mitochondrial membranes contain even lower amount of this lipid (Comte *et al*., 1976, Saks, *et al*., 1985). Importantly, the method can be applied to extremely small pieces of tissue that made it possible to study the mitochondrial function with very small samples: biopsy samples, tissues of expensive transgenic animals, cell cultures (Saks *et al*., 1998b).

The method of investigation of mitochondrial function in permeabilized preparation was validated using the following criteria: (1) well-preserved mitochondrial morphology; (2) complete permeabilization of the plasmalemma; (3) normal mitochondrial functions. Ultrastructural studies of permeabilized preparations by several groups (Saks *et al*., 1993; 1991; Penman, 1995; Lin *et al*., 1990, Kunz, 1993a) showed that digitonin — treated cardiomyocytes retained good overall morphology (Altschuld *et al*., 1985), mitochondria remained intact and retained the appearance of those in control, non-treated cells. The sarcomers of the digitonin-lysed cells were well aligned with only a slight thickening of the Z-lines.

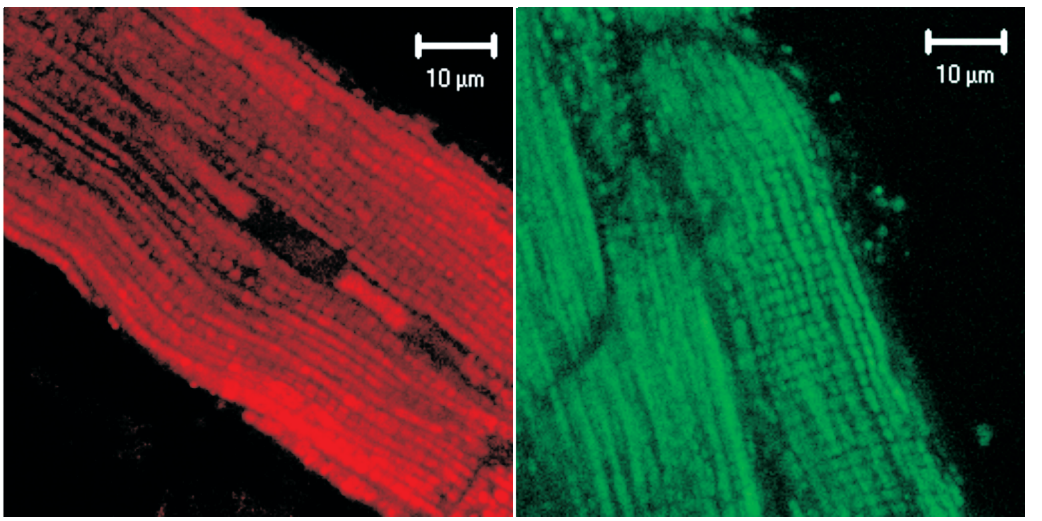


Figure 16. Confocal imaging of mitochondria in permeabilized skinned cardiac fibers using fluorescent probe. Left – membrane potential-sensitive fluorescent marker tetramethylrodamine ethyl ether, right – autofluorecenc of mitochondrial flavoproteins in fully oxidized state. Homogeneous labeling of all mitochondrial population in fibers is seen. Both preparations showed a very regular arrangement of "mitochondrial columns" in saponin permeabilized cells (From personal communications with T. Andrienko and A. Kuznetsov).

Electron microscopic investigation of saponin-skinned ventricular fibers (Veksler, 1987; Saks*, et al.*, 1993) also revealed the intact ultrastructure of mitochondria and sarcoplasmic reticulum, though the sarcolemma was completely removed (Fig. 16). Also, Cr significantly modulates the respiratory activity of skinned ventricular fibers at submaximal ADP concentrations. Penman group used treatment with 100 μg/ml saponin for 30 min to permeabilize smooth muscle cells and they showed that under these conditions not only outer mitochondrial membrane but also its multiple contacts with cytoskeleton are perfectly preserved (Penman, 1995; Lin *et al*., 1990).

Mitochondria in the saponin-skinned fibers are capable to use various substrates which suggest that all mitochondrial respiratory complexes are active (Kunz *et al*., 1993a; b). ADP stimulates respiration of permeabilized preparations manifold so that the acceptor control ratio is in the range of 5–7 for cardiac muscle of various species. Direct measurement of the ATP synthesis in digitonin permeabilized cells showed ATP/O ratios which were very similar to those obtained with isolated mitochondria using the same respiratory substrates (Ouhabi *et al*., 1994).

4.2. Respiratory characteristics of mitochondria in the cells *in vivo*

The cytochrome c test (Fig. 17 A) is used to investigate the state of the outer mitochondrial membrane before and after any treatment of the tissue, including isolated mitochondria. In the KCl medium cytochrome c dissociates from the outer surface of the inner mitochondrial membrane and if the outer mitochondrial membrane is damaged, it leaves mitochondrial intermembrane space, thus decreasing the rate of respiration (Saks *et al*., 1993). However, addition of the exogenous cytochrome c completely restores respiration under these conditions. Since KCI in high concentration dissociates also creatine kinase from mitochondrial inner membrane surface, it is not advised to use this solution to study the effects of creatine on respiration.

To investigate the intactness of coupling between mitochondrial CK and ANT the *creatine test* is used (Fig. 17 B, C) (Saks *et al*., 1995). Since this coupling is based on metabolic channeling of adenine nucleotides, any changes in membrane morphology, especially swelling of mitochondria and dissociation of CK by increased inorganic phosphate from the membrane may eliminate Cr stimulation of respiration and decrease the efficiency of control of respiration by the CK reaction. In fibers from the intact heart, addition of Cr in the presence of 0.1 mM ADP (submaximal concentration) activates respiration by activating production of ADP and PCr from mitochondrial ATP. In fibers from ischemic heart, when due to mitochondrial swelling the CK dissociates from the membrane, Cr stimulation is lost.

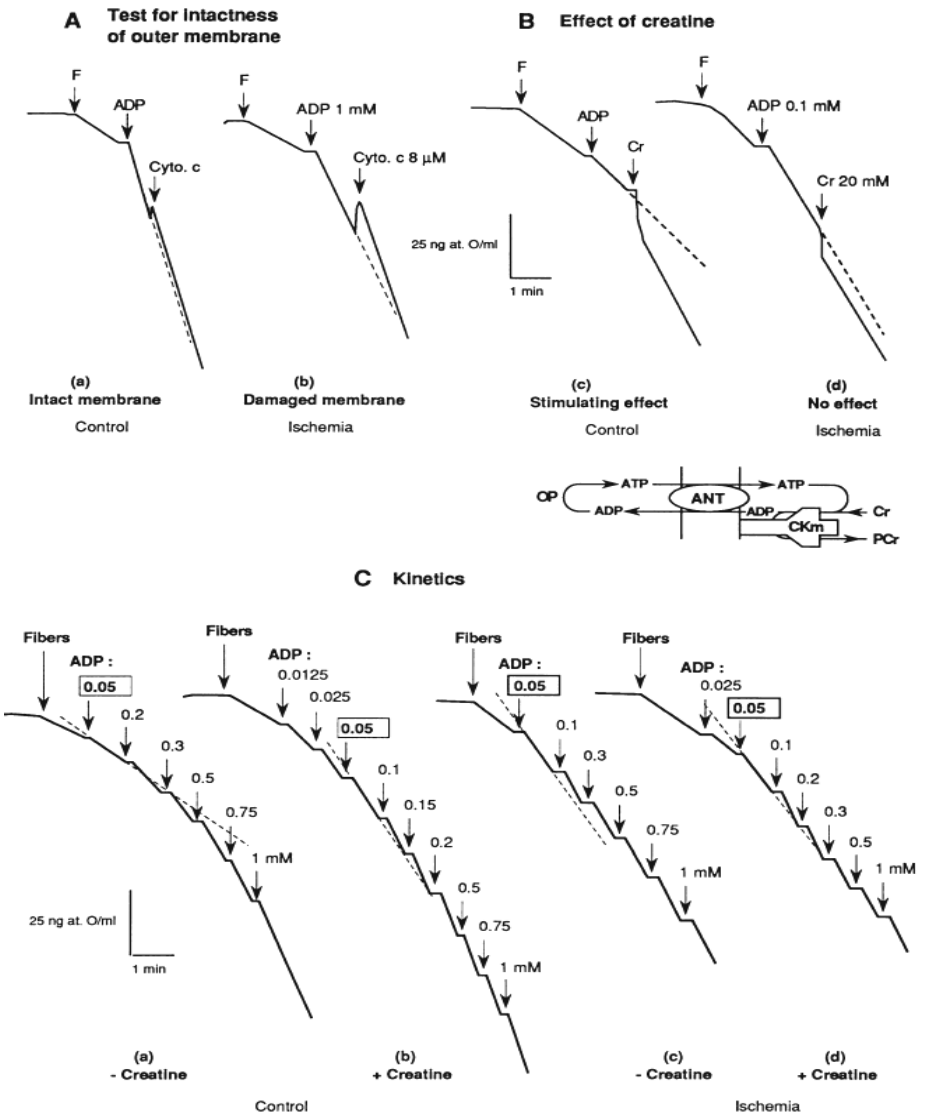


Figure 17. Oxygraphy recordings of the cardiac skinned fiber respiration.(A) Recording of a cytocrome c test for intactness of the outer mitochondrial membrane. ADP was added to final concentration of 1 mM for nearly maximal activation of respiration, then cytochrome c was added to final concentration of 8 μM. Control-fibers isolated from heart *in situ*; ischemia – the heart before isolation of fibers was kept for 1 h under normothermic total ischemia. (B) Recording of the Cr test for the functional state of coupled MitCK reaction in skinned fibers. ADP was added in concentration of 0.1 mM, then Cr in concentration of 20 mM. This is one of the most sensitive indicators of the ischemic damage of the heart (C). Recording of kinetics of regulation of respiration by ADP in skinned fibers from heart. Cr concentration was 20 mM (From Saks *et al*., 1998b, with permission).

4.3. Kinetics of ADP regulation of respiration *in situ*

The conventional explanation that the rate of respiration of mitochondria in intact cells is governed by the cytoplasmic ADP concentration according to a simple Michaelis–Menten type relationship is in disagreement with many experimental data (Saks *et al*., 1994; 1995; Balaban, 1990; Wan *et al*., 1993; Jeffrey and Malloy, 1992). In the skinned fibers in which mitochondria are located inside almost intact cellular structures, the affinity of mitochondria for ADP in oxidative tissues such as heart, *m. soleus* (also in permeabilized hepatocytes, brain homogenates and diaphragma muscle) apparent Km for ADP in regulation of OxPhos is surprisingly high, exceeding that for mitochondria *in vitro* by more than order of magnitude (Bygrave and Lehninger, 1967, Wiseman *et al*., 1996, Ouhabi *et al*., 1994; Saks *et al*., 1989; 1994; 1995). Exogenous ADP was characterized by very high apparent Km equal to 300–400 μM (Saks *et al*., 1989; 1991; 1993; 1995; 1998b; Kay *et al*., 1997). Veksler have found that in the skinned fibers of fast-twitch skeletal muscle, the affinity of mitochondria for ADP is high and equal to that of isolated mitochondria (Veksler *et al*., 1995). These observations clearly showed that mitochondrial function is principally differently regulated in these two types of muscles (i.e. is tissue specific) (See Fig. 18, Saks *et al*., 1998b). These observations showed also that the high Km value cannot be trivially explained by limited diffusion of ADP into the cell as suggested in some studies (Kongas, *et al*., 2002; Kongas and van Beek, 2002). All experimental and theoretical results give us the clear and strong arguments against this simple explanation (Alijev and Saks, 1997; Saks *et al*., 1991; 1997, Vendelin *et al*., 2000; Weiss and Korge, 2001; Fontaine *et al*., 1995). In skinned fibers from *m. soleus*, partial inhibition of respiration by NaN_3 did not decrease the apparent Km for ADP signifycantly, this excluding the possible explanation of low apparent affinity of mitochondria to ADP in these cells by its rapid consumption due to high oxidative activity and by intracellular diffusion problems (Kay *et al*., 1997). The affinity of mitochondria for ADP in skinned fibers can be increased in three ways: (i) by swelling of mitochondria and disruption of the outer membrane by hypoosmotic treatment (ii) by treatment of skinned fibers by proteolytic enzymes (iii) by isolation of mitochondria.

In slow-twitch muscles activation of coupled CK reaction by addition of Cr, which initiates ADP production in the mitochondrial intermembrane space, significantly deceased the Km for externally added ADP down to (70–100 μM) (Table 1). The results indicate tight functional coupling between MitCK and ANT in skinned soleus and heart muscle (Veksler *et al*., 1987; Saks *et al*., 1993; 1994). For skinned fibers from *m. gastrocnemius* the effect was not seen by the standard methods (oxygraphy). The effect of Cr was practically absent in the frog heart muscle (Kuznetsov *et al*., 1996).

In ischemic heart the most rapid cellular change is mitochondrial swelling, and this may result in disrupting of the outer mitochondrial membrane or

dissociation of CK from the inner mitochondrial membrane. This is why Cr stimulated respiration in skinned fibers is a very sensitive indicator of the extent of acute ischemic damage and correspondingly of the efficiency of the cardioplegic protection of the heart in cardiac surgery and preservation (Rossi *et al*., 1998).

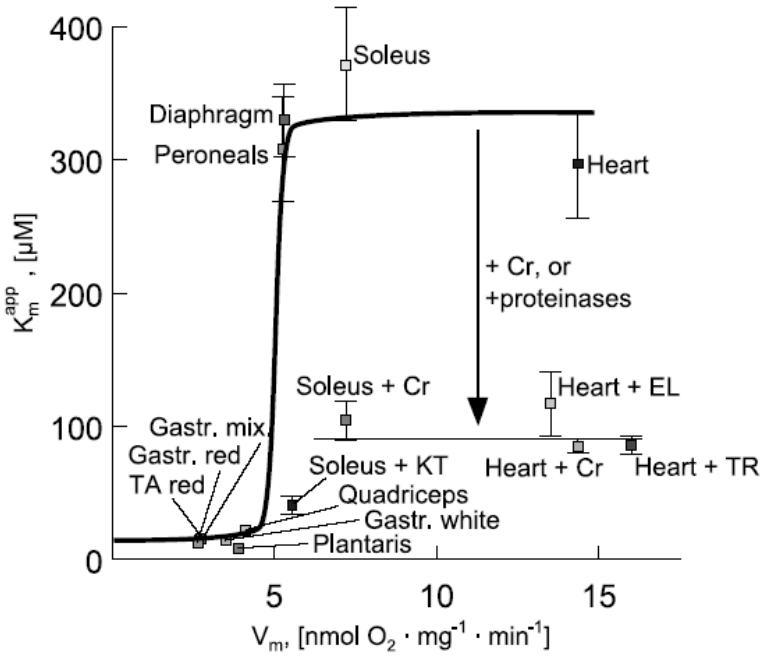


Figure 18. Tissue specificity of the affinity of the mitochondrial respiratory system for ADP. The affinities are low (apparent Km high) in heart, *m. soleus*, diaphragm, *m. peroneals* and increased by treatment with trypsin (TR), elastase (EL), chymotrypsin (KT) or by adding Cr. In all fibers from fast twitch skeletal muscles (*m. gastrocnemius* white, mixed, red, *quadriceps*, *m. tibialis anterior* and *plantaris*) the mitochondrial affinity for ADP for this substrate is very high (apparent Km very low) and not sensitive to trypsin or creatine additions. (From Saks *et al*., 1998b, with permission).

Hypoosmotic treatment resulted in the rupture of outer mitochondrial membrane and in the loss of exogenous cytochrome c, and both in skinned cardiac fibers and permeabilized hepatocytes the value of apparent Km for ADP was decreased in correlation with the extent of the rupture of the outer mitochondrial membrane. It is concluded that both in heart and liver cells *in vivo*, the ADP diffusion in the cells is retarded due to very low permeability of the outer mitochondrial membrane for ADP (Saks *et al*., 1995; 1996). The conclusion that porin pores, or channels, in the mitochondrial outer membrane limits the permeability for ADP in the cells *in vivo* was made on the basis of the results of

hypo-osmotic swelling resulting in rupture of the outer mitochondrial membrane and drastic decrease in apparent Km for ADP (Saks *et al*., 1993; 1994; 1995).

4.4. Proteolytic treatment and respiratory control

Limited proteolysis increases the affinity of mitochondria of the heart and slow-twitch skeletal muscles for ADP without destroying the outer mitochondrial membrane by trypsin.

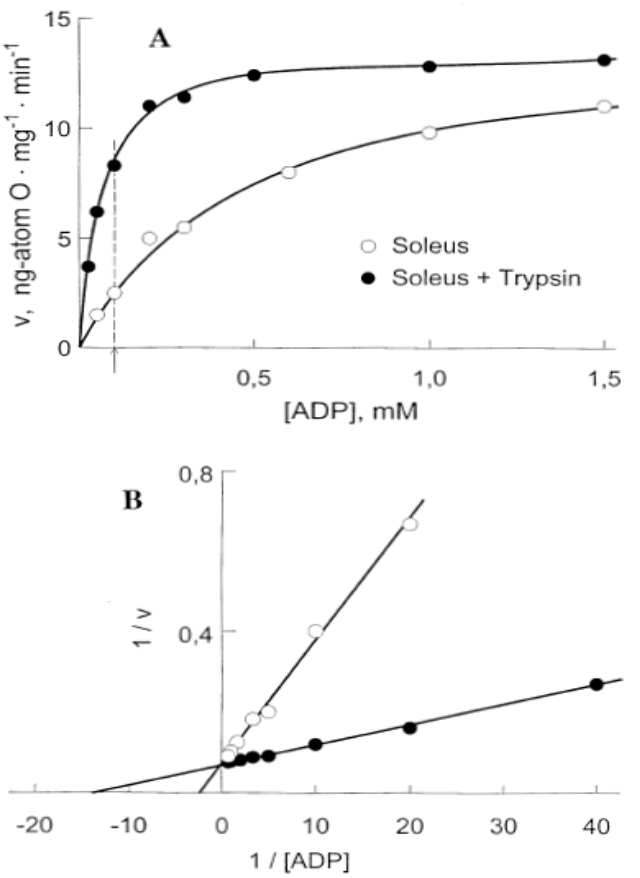


Figure 19. The effect of the treatment of skinned fibers from rat soleus muscles with trypsin on kinetics of regulation of respiration by ADP. Trypsin was used in concentration of 125 μg/ml for 15 min. (A) Changes in respiration rates as function of ADP concentration. (B) Treatment of data from Fig. 19A in double reciprocal plots. (From Saks *et al*., 1998b, with permission)

Treatment of the fibers with trypsin, chymotrypsin or elastase decreased the apparent Km for ADP (Fig. 18, 19) in cardiac and soleus muscle fibers to 40–98 μM without detectable alteration of the intactness of outer mitochondrial membrane (Kuznetsov, *et al*., 1996, Table 1), confirming the proposition that mitochondrial sensitivity to ADP in vivo is controlled by some cytoplasmic proteins. Phantom cardiomyocytes (cardiomyocytes without myosin) which contain mostly mitochondria, SR and cytoskeleton and retain the normal shape, showed also high apparent Km values for ADP (Kay *et al*., 1997). In liver tissue homogenates, trypsin treatment similarly decreased the Km value for ADP. These results show that ADP diffusion in hepatocytes may be retarded due to some unknown cytoplasmic trypsin-sensitive protein factor(s) which may be lost during isolation of mitochondria (Fontaine *et al*., 1995).

Slow freezing processes permit the observation of numerous strand-like connections between mitochondria and the myofibrillar surface within rat myocardial tissue (Rappaport, 1998). These results conform to the conclusion that the reason for observed high apparent Km for ADP in regulation of OxPhos heart and slow twitch skeletal muscle cells *in vivo* is low permeability of mitochondrial outer membrane porins but not diffusion problems of ADP into and inside the cells. Once again, it could be suggested that some proteins associated with the cytoskeleton control the permeability of the outer mitochondrial VDAC for ADP.

Table 1. Maximal oxygen consumption rates and Km^{app} (ADP) in different fibers. Mean ±SD are given for 6–12 experiments. Trypsin, chymotrypsin and elastase were used in concentration of 125 μg/ml (from Kuznetsov *et al*., 1996).

Fibre	Treatment	K_m^{app} (ADP)	V_{max}
		μM	nmol · min^{-1} · mg^{-1} N 2
Rat heart	none	297 ± 35	28.7 ± 1.1
	20 mM creatine	85 ± 5	28.0 ± 4
	ghost fibres	351 ± 32	46.7 ± 1.5
	trypsin	98 ± 8	26.0 ± 1
	chymotrypsin	143 ± 25	33.5 ± 2.0
	elastase	117 ± 24	27.0 ± 1.5
	creatine + chymotrypsin	120 ± 20	n.d.
Rat soleus	none	334 ± 46	12.2 ± 0.8
	20 mM creatine	105 ± 15	16.0 ± 2
	ghost fibres	320 ± 22	17.8 ± 0.4
	chymotrypsin	42 ± 8	11.8 ± 2
	elastase	42 ± 10	9.3 ± 1.0
	trypsin + trypsin inhibitor (Kunitz)	304 ± 25	10.7 ± 1
	trypsin	59 ± 6.0	n.d.
	creatine + trypsin	63 ± 3.0	n.d.
	hyposmotic treatment, phase I	351 ± 48	n.d.
	hyposmotic treatment, phase II	40 ± 8	n.d.
Rat gastrocnemius, white	none	14.4 ± 2.6	7.0 ± 0.5
	20 mM creatine	13 ± 2.0	5.4 ± 2.4
	trypsin	25 ± 4	8.9 ± 0.5
Rat gastrocnemius, red	none	12.8 ± 2.7	5.3 ± 0.3
	20 mM creatine	15 ± 8.5	7.6 ± 2.8
	trypsin	18 ± 2.8	n.d.
Rat quadriceps	none	22.3 ± 1.1	8.2 ± 1.2
	20 mM creatine	3.5 ± 0.7	6.2 ± 0.5
Rat plantaris	none	8.3 ± 5.4	7.8 ± 4.5
	20 mM creatine	20 ± 10	8.7 ± 2.4
Rat tibialis anterior red	none	15 ± 3	5.4 ± 0.3
Frog heart	none	94 ± 18	n.d.

5. Cytoskeleton

The cytoskeleton is a complex network of filaments and tubules which transmit mechanical and chemical stimuli within and between the cells (Coquet *et al*., 1997; Wang, 1994). It contributes substantially to the cell stability by anchoring subcellular structures, such as mitochondria, Golgi apparatus, nuclei and myofibrils. The cell cytoskeleton consists of three filament systems, micro-filaments, intermediate filaments and microtubules.

(I) Microfilaments are rod-like structures made of globular actin molecules and are common to all eukaryotic cells. Actin-containing microfilaments play an essential role in determining cell shape, and in cell locomotion and contractility. In the contractile unit of the skeletal muscle, i.e., the sarcomere, the thin filaments are interconnected and aligned at specialized structures, the Z-lines (Fürst and Gautel, 1995; Young *et al*., 1998). A common constituent of Z-lines, dense bodies, and the dense regions is α-actinin, an actin cross linking protein are involved in the arrangement of actin filaments (Geiger *et al*., 1981; Langanger *et al*., 1984; Chou *et al*., 1994; Young *et al*., 1998). Apart from α-actinin, several other components of the Z-disk have been identified (Fürst and Gautel, 1995; Young *et al*., 1998). Long chains of the actin molecules are intertwined in α helix to form individual microfilaments. At least six different types of actin are synthesized by vertebrate cells: four α-actins (skeletal, cardiac, vascular and enteric) are found in sarcomeric structures, while β and γ actins are thought to be predominantly cytoplasmic (Stromer, 1998). However, their amino acid sequences are highly conserved and they have similar properties. Actin filaments consist of two strands of polymerized actin monomers, twisted into a helix with 13.5 molecules per turn (Amos, 1985).

(II) The other group of filaments, intermediate filaments (IF) are not only the part of muscle cells but may stabilize organelles, like the nucleus, or may be involved in specialized junctions. They are distinguished from microfilaments by their size (8–10 nm) (Heins *et al*., 1993) and the fact that microfilaments are obviously motile. However, recent evidence indicates that IF may also have dynamic properties. There are five different types of intermediate filaments (http://www.cytochemistry.net/Cell-biology/intermediate_filaments.htm**)**:
Types 1 and 2. Acidic keratin and basic keratin, respectively**.** Produced by different types of epithelial cells (bladder, skin, etc.). **Type 3.** IF are distributed in a number of cell types, including: vimentin in fibroblasts, endothelial cells and leukocytes; desmin in muscle; glial fibrillary acidic factor in astrocytes and other types of glia, and peripherin in peripheral nerve fibers. **Type 4.** Neurofilament (H (heavy), M (medium) and L (low)). Another type 4 is "internexin" and some nonstandard 4's are found in lens fibers of the eye (filensin and phakinin). **Type 5** are the lamins which have a nuclear signal sequence so they can form a filamentous support inside the inner nuclear

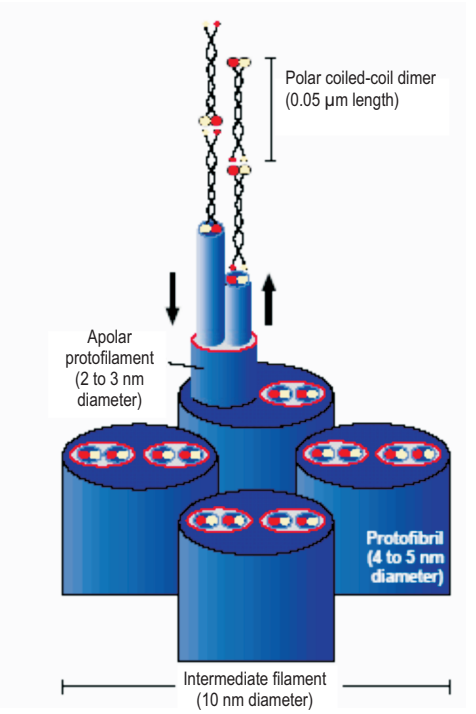


Figure 20. Structural model of an IF. Polar dimers of IF subunits form staggered antiparallel tetramers that associate longitudinally and laterally into apolar protofilaments 2 to 3 nm in diameter and protofibrils 4 to 5 nm in diameter. Two to four protofibrils (four are drawn here) associate to yield a coiled-coil lattice composed of approximately 16 to 32 polypeptides in cross section (From Fuchs and Cleveland, 1998, with permission).

membrane. Lamins are vital to the re-formation of the nuclear envelope after cell division.

IF associated proteins may bind filaments in cross linked (to improve stability), or they may bind the filaments to other structures. Despite their diversity, members of the IF superfamily share a common structure: a dimer composed of two α-helical chains oriented in parallel and intertwined in a coiled coil rod (Fig. 20). This mechanism of dimerization through coiled-coil interaction is now universally found throughout biology. The highly conserved ends of the IF rod associate in a head-to-tail fashion, and mutations in these rod ends have deleterious consequences for the assembly process of most if not all IF proteins (Albers and Fuchs, 1987; Letai *et al*., 1992; Geisler *et al*., 1983). The association of dimers results in linear arrays, four of which associate in an antiparallel, half-staggered manner to produce protofibrils, and three to four protofibrils intertwine to produce an apolar intermediate filament 10 nm in diameter (Fuch and Weber, 1994). Although IFs share similar structures, their properties can be quite unique.

(III) Microtubules are conveyer belts inside the cells. They move vesicles, granules, organelles like mitochondria, and chromosomes via special attachment proteins. They also serve a cytoskeletal role. Structurally, they are linear polymers of tubulin which is a globular protein. These linear polymers are called protofilaments. The tubulin family now includes α- and β-tubulin, which are the main subunits of eukaryotic microtubules; γ-tubulin, which nucleates these microtubules and regulates their dynamics at the minus end (Joshi, 1994; Oakley, 1995); and FtsZ, a prokaryotic homolog of the tubulins that is the major cytoskeletal protein in bacterial cell division (Erickson, 1995). Two new members of the tubulin family, δ- and ε-tubulin, have been inferred from sequences in databases (Burns, 1995), but so far the proteins have not been identified. Both α/β tubulin and FtsZ (Erickson, 1996) assemble into straight protofilaments that can associate further to make 2-D protofilament sheets. The protofilament sheet of α/β tubulin makes the microtubule wall. The α and β tubulin families consist of different isotypes, which are highly conserved, differentially expressed, and post-translationally modified (Little and Seehaus, 1988; Mandelkow and Mandelkow, 1995). The exchangeable GTP bound to the β-subunit is hydrolyzed to GDP as a result of assembly. Tubulin liganded to GDP is inactive for microtubule assembly and easily forms rings related to the curled protofilaments of depolymerizing microtubule ends (Melki *et al*., 1990; Diaz, *et al*., 1994). Two major classes of proteins (MAPs) interact with microtubule polymers: molecular motors and microtubule-associated proteins, which in turn are regulated by phosphorylation. In contrast with motor molecules, MAPs bind to microtubules in a nucleotide-independent fashion. They copolymerize with microtubules through cycles of assembly and disassembly, stimulate their nucleation, and stabilize the polymer (Olmsted, 1991; Schoenfeld and Obar, 1994; Mandelkow and Mandelkow, 1995).

5.1. The cytoskeleton in muscle cells

The proteins which contribute to cell shape, mechanical resistance, and morphological integrity of cardiomyocytes can be subdivided on the basis of their structural and functional properties into four different groups (Kostin *et al*., 1998):

1. Sarcomeric skeleton: titin, C-protein, a-actinin, myomesin, and M-protein.
2. "True" cytoskeletal proteins: tubulin, desmin and actin.
3. Membrane-associated proteins: dystrophin, spectrin, talin, vinculin, ankyrin.
4. Proteins of the intercalated disc: desmosomes consisting of desmoplakin, desmocollin, desmoglein and desmin adherens junctions with N-cadherin, the catenins and vinculin and gap junctions with connexin.

The action of the cytoskeleton as a stabilizing force and as mechanotransductor is supported by membrane-associated proteins, especially dystrophin that binds to both intracellular actin and extracellular laminin (Klietsch *et al*., 1993). A close integrin-cytoskeleton linkage system exists and allows cells to respond to physical and biochemical influences exerted by extracellular matrix. The cytoskeleton forms connections with the extracellular matrix via membrane-spanning integrins at sites close to the Z-line known as costameres. When the matrix resists movement, the linkage to the cytoskeleton is strengthened via increased number of integrins (Choquet *et al*., 1997). Of the components of the cytoskeleton, it is actin that has been assigned the primary role in this respect, although microtubules and intermediate filaments may also be involved (Wang *et al*., 1993; Maniotis *et al*., 1997). Many of the proteins with which integrins associate at the costamere are actin binding proteins e.g. vinculin, α-actinin, talin, paxillin (Borg *et al*., 2000; Samuel *et al*., 2000). These proteins maintain the organisation of cytoskeletal actin in the cell by cross-linking microfilaments, providing links with the sarcolemma, and regulating filament length (Raman and Atkinson, 1999). Other membrane spanning proteins such as β-dystroglycan form connections with the actin cytoskeleton via dystrophin (see Kaprielian *et al*., 2000). For example, Rybakova *et al*. (2000) showed a costameric patterning of cytoskeletal G-actin in adult skeletal muscle which was absent in the dystrophin-deficient mouse. In slow-twitch muscle cells, the absence of dystrophin is associated with the rearrangement of the intracellular energy and feedback signal transfer systems between mitochondria and ATPases (Braun *et al*., 2001).

In multiple ultrastructural studies of the cells by using electron microscopy, the connections between mitochondria and cytoskeleton elements have been observed since 1967 (Bucley and Porter, 1967; Lazarides and Granger, 1982; Watkins *et al*., 1987; Lockhard and Bloom, 1993; Leterrier *et al*., 1994; Bereiter-Hahn and Voth, 1994; Penman, 1995; Rappaport *et al*., 1998). The results of these work show many connections between cytoskeleton and mitochondrial membranes, which are taken to be important for localization of

mitochondria in the cells and structural organization of the cellular systems, also these contacts may be important for control of some important mitochondrial function such as affinity to cytoplasmic ADP as discussed above.

5.1.1. Cardiac actin cytoskeleton

In both neonatal and adult cultured myocytes distinct sarcomeric and nonsarcomeric actin can be seen (Sadoshima *et al*., 1992; Messerli and Perriard, 1995; Larsen *et a*l., 1999; 2000). Indeed it seems that these non-sarcomeric structures are necessary for cell spreading and the generation of new myofibrils (Rothen-Rutishauser *et al*., 1998). However immunocytochemical imaging of actin in isolated adult myocytes reveals uniquely sarcomeric patterning (Messerli and Perriard, 1995; Kostin *et al*., 1998), even when using antibodies to β-actin, one of the cytosolic actin isomers observed sparse cortical cytoskeletal actin in the adult rat myocardium, which was increased by hypertrophy (Yang *et al*., 2002). They also found that either disrupt (cytochalasin D) or stabilize (phalloidin) the subsarcolemmal non-myofibrillar actin microfilament component alter action potential configuration. The evidence for an important role of the actin cytoskeleton in modulating electrical activity of the heart is very convincing in neonatal and cultured myocytes in which an actin microfilament network can be clearly visualized. Despite the lack of clear evidence for modulation of the action potential by cytoskeletal actin, there are now many reports of effects upon cardiac ionic currents where the logical conclusion is modulation of channel activity by the non-sarcomeric actin cytoskeleton (Calaghan *et al*., 2004). There exist differences between costameric (cytoskeletal) actin in cultured cells and in the intact myocardium (Calaghan *et al*., 2004). In cells cultured on extracellular matrix substrates, actin is always present in the focal adhesion sites at the costamere; however it has not been detected as a part of the extracellular matrix-integrin axis in the intact myocardium (Borg *et al*., 2000).

Cytochalasin-D is the most commonly used pharmacological tool to study the actin cytoskeleton. Cyto-B has also been used to disrupt the actin cytoskeleton (Dick and Lab, 1998). Other agents which can be used to disrupt actin include latrunculin A, DNAase I, gelsolin and cofilin.

Thus the structure of the actin cytoskeleton in the adult myocyte has not been well characterised, and it is clear that there are marked differences between the density and distribution of actin microfilaments in cultured or neonatal cardiac myocytes compared with that in adult myocytes, or in the intact myocardium. Because of difficulties in visualizing cytoskeletal actin, verification of the effects of pharmacological intervention or alterations seen in pathological conditions is problematic (Kostin *et al*., 1998).

5.1.2. Microtubules in muscle cells

Microtubules are hollow protein cylinders of α- and β-tubulin heterodimers about 25nm in diameter (Goldstein and Entman, 1979; Rappaport and Samuel, 1988) aligned predominantly along the longitudinal axis of the adult myocyte (Kostin *et al*., 2000).The T-system is an essential organelle of the working ventricular cardiomyocytes; while the volume density of this system is 1–2%, it represents about one third of the entire cell membrane area (Severs *et al*., 1985). In both skeletal and cardiac muscle the T-system is necessary for the rapid propagation of the excitation impulse throughout the muscle fiber (Sommer, 1995).

A constant turnover of microtubules by polymerization and depolymerization takes place. In cardiomyocytes, only 30% of total tubulin is present in the polymerized form as microtubules where as 70% occurs as non-polymerized cytosolic protein (Tagawa *et al*., 1998). Microtubules are found in association with GTP-binding proteins such as Gi and Gs (Rasenick *et al*., 1981; 1990), MAPs that promote microtubule stability (predominantly MAP4 in cardiac muscle (Olmsted, 1986; Sato *et al*., 1997)), cell matrix focal adhesion molecules (Kostin *et al*., 1998), Tau protein, kinases (Pitcher *et al*., 1998), and molecular motors such as kinesin (Olmsted, 1986; Liao and Gundersen, 1998). Microtubules also associate with other components of the cytoskeleton such as actin (Cunningham *et al*., 1997) and IF (Gurland and Gundersen, 1995). MAPs has been shown to reorganize both actin microfilaments and microtubules (Cunningham *et al*., 1997).

The most popular agents used to manipulate the microtubule cytoskeleton are colchicine, which causes disruption of the microtubules, and taxol, which causes their proliferation and stabilization. Immunocytochemistry and Western blotting studies have shown that exposure of adult cardiac myocytes to colchicine leads to a decrease in intact microtubules and an increase in free tubulin (Tsutsui *et al*., 1994; Calaghan *et al*., 2001; Kerfant *et al*., 2001), whereas taxol (10 mM for 2–4 h) has the opposite effect (Tsutsui *et al*., 1994; Howarth *et al*., 1999). Taxol has been shown to decrease the $[Ca^{2+}]_i$ transient in rat ventricular myocytes (Howarth *et al*., 1999). This reduction in the $[Ca^{2+}]_i$ transient was due, in part at least, to reduced $[Ca^{2+}]_i$ release from the SR (Howarth *et al*., 1999). The publication presenting an analysis of $[Ca^{2+}]_i$ transient during microtubule alteration in adult cardiac myocytes is report of Kerfant (2001), in which an increase in $[Ca^{2+}]_i$ transient after longer colchicine treatment was demonstrated.

Consistent with a lack of effect upon the $[Ca^{2+}]_i$ transient, in the absence of prior microtubule proliferation, disruption of the microtubular cytoskeleton by colchicine does not significantly modulate cardiac contractility in adult cardiac muscle or myocytes (Tsutsui *et al*., 1994; Ishibashi *et al*., 1996; Tagawa *et al*., 1998; Takahashi *et al*., 1998; Hongo *et al*., 2000; Calaghan *et al*., 2001). However, in apparent contrast to these studies, it has also been reported that in rat ventricular myocytes colchicine decreased the amplitude of $[Ca^{2+}]_i$ sparks

and increased the amplitude of the global $[Ca^{2+}]_i$ transient (Gomez *et al*., 2000; Kerfant *et al*., 2001)

Microtubules are linked to the β-adrenergic pathway in cardiac muscle (Palmer *et al*., 1998) and have also been implicated in post-receptor β-adrenergic signaling (Rasernick *et al*., 1990). The role of microtubules in the regulation of the β-adrenergic signaling pathway in adult cardiac muscle is a subject of debate (Calaghan, 2004). Microtubules and mitochondria seem to be in close connection, one might logically expect a specific junction between components of the mitochondrial membrane and components of microtubules. Microtubular-mitochondria interactions appear specific and of general relevance to all cells (Bernier-Valentin and Rousset, 1982). The results of Saetersdal *et al*., (1990) indicate the presence of a specific β-tubulin binding to the outer mitochondrial membrane that probably also involves microtubule based translocators and/or MAPs. It appears that specialized domains of the mitochondrial membrane are involved in the MAP-mediated association of mitochondria to microtubules (Leterrier *et al*., 1994).

The microtubular cytoskeleton has been linked with various pathological conditions. Disruption of the microtubular network has been reported in ischaemia (Hori *et al*., 1994; Hein *et al*., 1995), irreversible cell damage may be associated with collapse of the microtubule cytoskeleton (Iwai *et al*., 1990). Proliferation of the microtubules with taxol has been reported to protect against hypoxia/re-oxygenation injury (Skobel and Kammermeier, 1997), whereas the microtubule disruptor colchicine has been shown to abolish ischemic pre-conditioning (Sharma and Singh, 2000a, b).

5.1.3. Desmin

Desmin forms the major component of cardiac IFs which forms a physical link between the nucleus, contractile proteins (by surrounding the Z-discs), sarcolemma and extracellular matrix via costameres. They also have the potential to associate with other organelles including mitochondria and the SR (Capetanaki, 2002) (Fig. 21). Their cellular distribution makes them an ideal candidate for mechanical signaling into and out of the cell.

Desmin molecule is organized into three domains: a highly conserved α-helical rod-like domain flanked by non-helical head and tail domains. The rod consists of four α-helical segments, 1A, 1B, 2A and 2B, interrupted by linkers (Fuchs and Weber, 1994). Mice lacking desmin show severe disruption of muscle architecture in myocardium and skeletal muscles (Li *et al.*, 1996). Desmin mutations cause abnormal filament assembly (Munos-Marmol *et al*., 1998; Park *et al*., 2000), disruption of a preexisting endogenous filament network produced by other IFs (Sjoberg, 1999) and accumulation of insoluble chimeric intracellular aggregates (Li and Dalakas, 2001; Wang *et al*., 2001).

IFs are prominently associated with mitochondria, and this association has been demonstrated in a number of cell types (Capetanaki, 2002; Rappaport *et al*., 1998). The absence of desmin leads to loss of proper mitochondrial distribution, number, morphology, and function (Milner *et al*., 2000). Furthermore, extensive calcification in desmin-null hearts (Mavroidis and Capetanaki, 2002; Weisleder *et al.*, 2004) could be either directly linked to loss of normal Ca^{2+} homeostasis, or could be the consequence of extensive cell death that might have originated by a Ca^{2+}–independent way linked to mitochondria. In order to investigate the possible role of desmin filaments in control of mitochondria transgenic mice deficient in desmin have been used (Kay *et al*., 1997). Phantom cardiomyocytes which contain mostly mitochondria, SR and cytoskeleton in desmin retain the normal shape, showed also high apparent Km values for ADP. Therefore, they are probably the most suitable system for studies of cellular factors which control mitochondrial function in the cells in vivo. In these phantom cells anti-desmin antibodies did not change the kinetics of respiration regulation by ADP. However, in skinned fibers from the heart and m. soleus of transgenic desmin-deficient mice some changes in kinetics of respiration regulation by ADP were observed (Kay *et al*., 1997). Morphological observations by electron microscopy confirmed the existence of two distinct cellular populations in the muscle cells of desmin-deficient mice. Desmin itself does not display this type of control of mitochondrial porin pores, but its absence results in appearance of cells with disorganized structure and of altered mitochondrial population probably lacking this unknown VDAC controlling protein.

5.1.4. Plectin

Plectin, a high molecular weight (500 kDa) protein, is abundantly expressed in a wide variety of mammalian tissue and cell types, and has been proposed to function as a general cross-linking element of the cytoskeleton (Foisner and Wiche, 1991) (Fig. 22). Actually, the precise size predictions for full length plectin isoforms vary from 507000 to 527000, depending on several putative first coding exons (Wiche, 1998).

Electron microscopy of single molecules and secondary structure predictions based on the cloning and sequencing of rat plectin (Wiche *et al*., 1991; Elliott *et al*., 1997) revealed a multidomain structure comprising a ~200-nm-long central α-helical coiled–coil rod structure flanked by large globular domains. A similar domain organization has been found in proteins that share partial sequence homology with plectin, such as desmoplakin (Green *et al*., 1990), and envoplakin (Ruhrberg *et al*., 1996). The amino-terminal domain of plectin contains a highly conserved actin-binding domain of the type found in spectrin, dystrophin, and other related proteins (Elliott *et al*., 1997), whereas the carboxyl

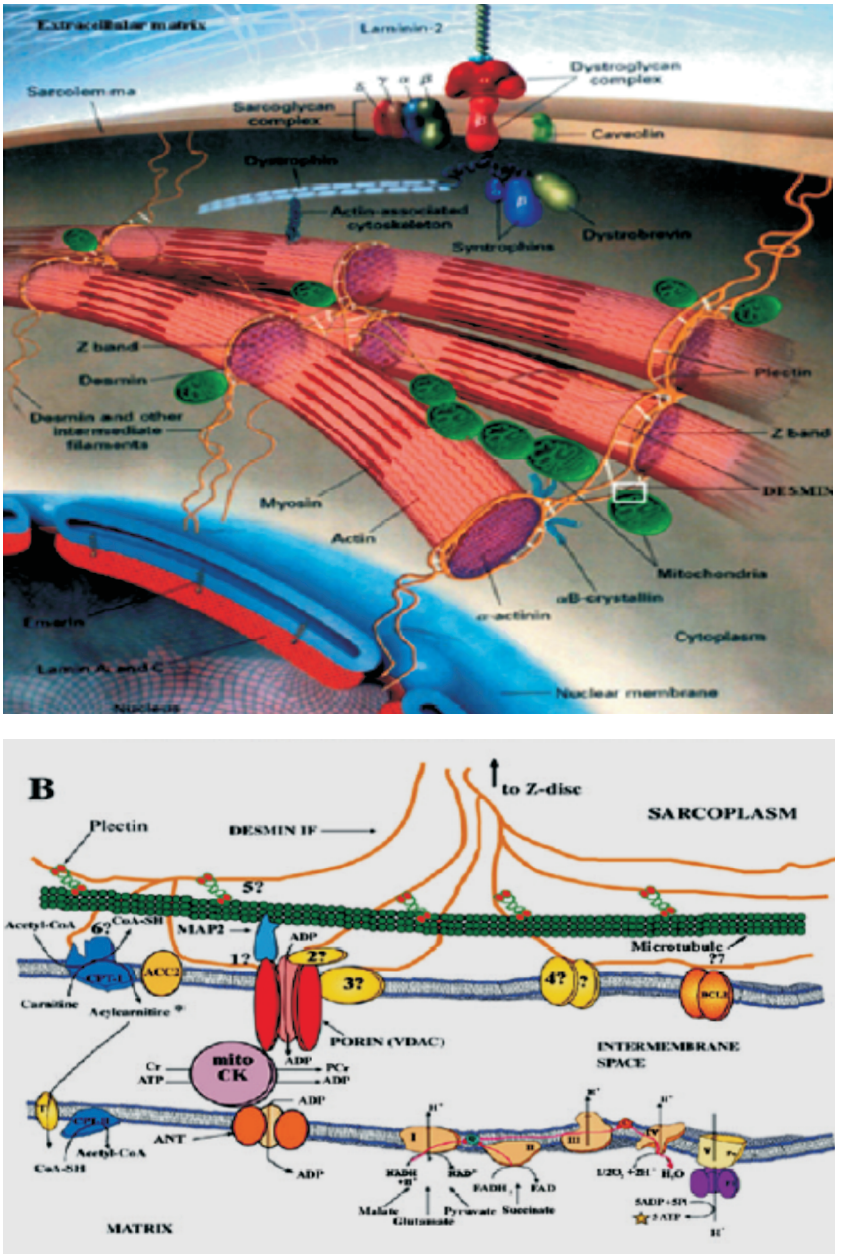


Figure 21. The cytoskeleton in cardiac cells. Schematic representation of desmin IF scaffold in striated muscle **(A)**, and potential associations with mitochondria **(B)**. **(A)** The desmin IF lattice (orange) surrounds the Z discs, interconnects them, and links the entire contractile apparatus to different membranous compartments and organelles including the sarcolemma, mitochondria, and nucleus. This allows for the formation of a continuous network connecting the extracellular with the nuclear matrix. Potential connections with SR and T tubules system are not shown here. The white square shows a hypothetical area of mitochondrial-desmin association elaborated in (B). **(B)** Schematic diagram outlining how the desmin IF cytoskeleton could associate with mitochondrial contact sites and potentially influence membrane stability and mitochondrial function. These sites are dynamic structures that involve a fusion of the inner and outer mitochondria membrane (fusion not shown here), and are key participants in protein transport, energy coupling with the cytosol via formation of PCr, and uptake of fatty acids for oxidative metabolism (From Capetanaki *et al*., 2002, with permission).

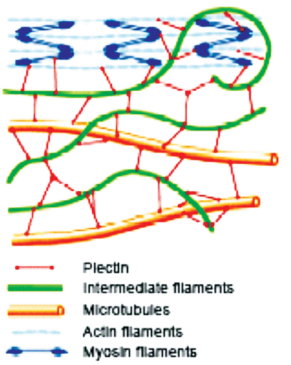


Figure 22. Model suggesting that plectin mediates linking between IFs, microtubules, actin, myosin, and membrane-bound adhesion sites (focal contacts or plasma membrane components). (From Fuchs and Cleveland, 1998, with permission)

terminus harbors one or more IF-binding sites, including at least one for cytokeratins (Nikolic *et al*., 1996).

The strategic localization of plectin at the cytoskeleton–plasma membrane interface and its versatile binding capacities suggest that plectin could strengthen cells against mechanical stress, both along their surface and at their internal cytoskeleton anchorage sites. Plectin can associate with not only all three major cytoskeletal filaments comprising IFs, microtubules, and microfilaments, but also many membrane and cytoskeletal proteins.

The *in vivo* association of plectin with desmin, vinculin, and actin was demonstrated by immunoprecipitation experiments (Hijkata *et al*., 2003). Immunofluorescence analysis also revealed that plectin was co localized with desmin at the periphery of Z-discs (Fig. 23). The plectin localization around Z-discs was constantly maintained irrespective of the contracted or extended state of the muscle fibers, suggesting either direct or indirect association of plectin with Z-discs (Hijikata *et al*., 1999). Plectin is associated with desmin IF´s linking myofibrils to mitochondria at the level of the Z-disc and along the entire length of the sarcomere. The localization of plectin label at the mitochondrial membrane itself was consistent with a putative linker function of plectin between desmin IFs and the mitochondrial surface. In mitochondrion-rich muscle fibers, both plectin and desmin were part of an ordered arrangement of mitochondrial side branches, which wound around myofibrils adjacent to the Z-discs and were anchored into a filamentous network transversing from one fibril to the other (Reipert, 1999). Its localization at the mitochondrial membrane accounts for a higher binding found in muscle fibers and suggests, as a cytolinker protein, an important role in the positioning and shape formation of mitochondrial organelles and mechanical stabilization of cells.

The important role of plectin as a versatile cytolinker protein is also evident from the phenotypic analysis of plectin gene knockout mice which died 2–3 days after birth due to severe skin blistering and clearly showed abnormalities in muscle and heart. The overall hallmark of the plectin (−/−) phenotype in skeletal muscle observed at the light microscopy level was an increased number of necrotic muscle fibers. At the ultrastructural level, necrotic changes involving focal loss of myofilaments of various degrees were observed in ~20% of the muscle fibers (Andra *et al*., 1997).

Among other cytoskeletal proteins assumed to have a role in the development and integrity of sarcomeric cytoarchitecture, such as vinculin, spectrin, and dystrophin, vinculin expression was found to be the one affected most significantly by plectin deficiency. Focal disruptions of sarcomeres affecting Z-lines and adjacent myofibrils were frequently noticeable on longitudinal sections obtained from plectin deficient mice. In affected areas, the Z-lines have lost their tight arrangement, displaying a poorly focused, smearing and streaming appearance. The streaming of Z-lines s also observed in cardiac muscle. In addition, sarcomeres were found frequently to be loosely and irregularly arranged in these cells (Andra *et al*., 1997).

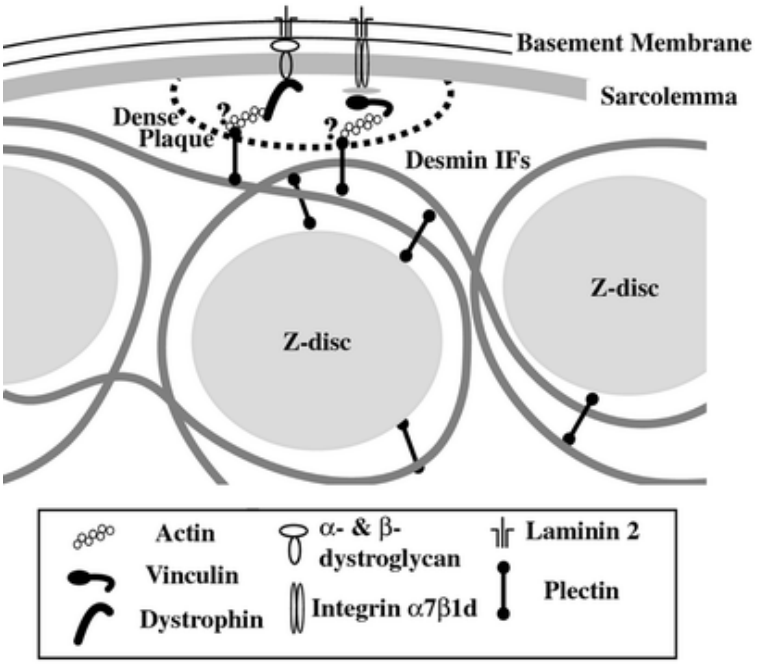


Figure 23. A schematic drawing of the transverse cytoskeletal networks from myofibrils to the basement membrane. Desmin IFs, via plectin, connect with Z-discs, while they anchor onto subsarcolemmal dense plaques through plectin. In the subsarcolemmal dense plaques, plectin may interact, via actin, with dystrophin and vinculin. Question marks implicate the possibility that unidentified molecules other than actin may also mediate interactions between plectin and dystrophin or vinculin. Dystrophin binds, via α- and β-dystroglycan on the outer surface of the sarcolemma. On the other hand, vinculin may interact with integrin α7β1d, which can also bind laminin-2 (From Hijikata, 2003, with permission).

Patients lacking plectin suffer from epidermolysis bullosa simplex associated with muscular dystrophy (McLean *et al*., 1996; Gache *et al*., 1996a; Smith *et al*., 1996; Pulkkinen *et al*., 1996), a severe skin blistering disease combined with muscular dystrophy and in some cases respiratory distress. The disease results from a homozygous nonsense mutation in the plectin (PLEC1) gene leading to a premature stop codon (CGA to TGA) and decay of the aberrant plectin messenger RNA and associated with an impaired expression of plectin (Gache *et al*., 1996b).

Due to their large size, multiple binding abilities, and association with internal IFs and peripheral subplasma membrane structures and junctional complexes, plectin molecules may provide a structural platform for the localized assembly of multi-component protein machineries, including regulatory factors and molecules forming signaling cascades.

AIM OF THE THESIS

A number of studies including those from our laboratory have shown that striated muscle cells are highly organized structures that include compartmentalized enzyme systems and metabolite fluxes which ensure synchronized energy consumption in contractile apparatus and ATP resynthesis in mitochondria.

The data available in the literature show that the mitochondrial function is regulated very differently *in vitro* and *in vivo*. *In vitro* the affinity of the respiratory system for ADP is high (Km is 10–20 μM). *In vivo* the Km^{app} is 300–400 μM and the parameter is controlled by cellular structures. The reason for these differences is not known. Therefore in this study we investigated the next problems:

1) The importance of mitochondrial outer membrane using the proapoptotic protein Bax.
2) The kinetics of regulation of respiration by exogenous and endogenous ADP.
3) The effect of cytoskeleton in regulation of ATP production in mitochondria.
4) The effect of Ca^{2+} on the mitochondrial respiration in situ.

These studies have recently resulted in formulation of the concept of intracellular energetic units (ICEU). The concept claims that the muscle cell is organized as an autonomic unit in which the energy is produced and utilized without the need of its transporting to long distances. Briefly, it is suggested that the contractile apparatus, mitochondria and calcium-supplying sarcoplasmic reticulum of a cardiac sarcomere enveloped by cytoskeletal network functions as a single synchronized entity.

MATERIALS AND METHODS

1. Animals

Male Wistar rats weighing 300–350 g and wild type C57BL/6 mice were used in experiments. Animals were housed five per cage at constant temperature (22±1ºC) in environmental facilities with a 12:12h light-dark cycle schedule and were given standard laboratory chow and tap water ad libitum. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85–23, revised 1985).

2. Solutions

Composition of the solutions used for preparation of skinned fibers and for oxygraphy was based on the information of the ionic content in the muscle cell cytoplasm (Godt and Maughan, 1988; Veksler *et al.*, 1987). In some cases the modified solutions were used to decrease the free Ca^{2+} concentration. The choice of solutions depends on the aim of the work.

Solution A (classical) contained, in mM: CaK_2EGTA 2.77, K_2EGTA 7.23, $MgCl_2$ 6.56, dithiothreitol 0.5, potassium 2-(N-morpholino)ethanesulfonate (K-Mes) 50, imidazole 20, taurine 20, Na_2ATP 5.3, PCr 15, pH 7.1 adjusted at 25°C.

Solution A (modified) contained, in mM: CaK_2EGTA 1.81, K_2EGTA 8.19, $MgCl_2$ 6.56, dithiothreitol 0.5, K-Mes 50, imidazole 20, taurine 20, Na_2ATP 5.3, PCr 15, pH 7.1 adjusted at 25°C.

Solution A (modified) contained, in mM: CaK_2EGTA 1.9, K_2EGTA 8.1, $MgCl_2$ 9.5, dithiothreitol 0.5, K-Mes 50, imidazole 20, taurine 20, Na_2ATP 2.5, PCr 15, pH 7.1 adjusted at 25°C.

Solution B contained, in mM: CaK_2EGTA 2.77, K_2EGTA 7.23, $MgCl_2$ 1.38, DTT 0.5, K-Mes 100, imidazole 20, taurine 20, K_2HPO_4 3 and pyruvate 5 (or glutamate 5), malate 2, pH 7.1 adjusted at 25°C.

Solution B (modified) contained, in mM: CaK_2EGTA 1.81, K_2EGTA 8.19, $MgCl_2$ 4.0, DTT 0.5, K-Mes 100, imidazole 20, taurine 20, K_2HPO_4 3 and pyruvate 5 (or glutamate 5) and malate 2, pH 7.1 adjusted at 25°C.

Solution C contained, in mM: potassium Hepes, 50; $MgCl_2$, 10; ATP, 10; DTT, 0.5; taurine, 20; potassium Mes 80; (pH 7,1).

Solution D contained, in mM: KCl, 800; potassium Hepes, 50; $MgCl_2$, 10; ATP, 10; DTT, 0,5 taurine, 20; (pH 7,1).

Solution KCl contained in mM: KCl 125, Hepes 20, glutamate 4, malate 2, Mg-acetate 3, KH_2PO_4 5, EGTA 0.4 and DTT 0.3, pH 7.1 adjusted at 25°C and 2 mg of BSA per ml was added.

Sucrose solution for respirometry contained, in mM: sucrose 240, EGTA 1, HEPES 50, and KH_2PO_4 3, pH 7.2; BSA 2 mg/ml, glutamate 5 mM, and malate 2 mM.

Reagents. All reagents were purchased from Sigma (USA).

3. Calculation of free Ca^{2+} and Mg^{2+} ion concentrations

Calculations of the composition of EGTA-Ca buffer were made according to Fabiato and Fabiato (Fabiato and Fabiato, 1979) first for 1.878 mM of total Ca^{2+} concentration. For our calculations, dissociation constants of complexes of Mg^{2+} with ADP and ATP were taken from (Phillips *et al*., 1966) and (Saks *et al*., 1975). 10 mM EGTA, 2.26 mM ATP were used as ligand concentrations and 9.5 mM Mg^{2+}, 1.878 or 2.77 mM Ca^{2+} were used for metals for calculations for solution A. For solution B we decreased the concentration of Mg^{2+} to 4 mM and added 3 mM phosphate. Concentration of free Ca^{2+} in case of 1.878 mM total Ca^{2+} was found for solution A 1.11×10^{-7} M and solution B 1.04×10^{-7} M. In case of 2.77 mM^{-1} total Ca^{2+}, free Ca^{2+} for solution A was 1.84×10^{-7} M l^{-1} and for solution B 1.72×10^{-7} M confirming our previous rough predictions.

To increase the free Ca^{2+} concentration up to 3 µM, the total EGTA concentration in solution B was kept constant at 10 mM and total Ca^{2+} concentration changed by adding calculated aliquots of stock solution of 270 mM $CaCl_2$. The necessary total Ca^{2+} concentrations for achieving corresponding free Ca^{2+} concentrations were calculated using the WINMAXC program according to the scheme described above. Analysis of the calculations allowed us also to use simpler empirical formula:

$$[Ca]_{total} = \frac{a \cdot [Ca]_{free}}{b + [Ca]_{free}}, \tag{4}$$

where a = 10.0945 ± 0.01406 and b = 0.4574 ± 0.0021, for these coefficients, $[Ca]_{free}$ is given in µM, $[Ca^{2+}]_{total}$ in mM.

At 21°C, pH 7,1 and ionic strength 0.175 M the total Ca^{2+} concentrations, to obtain the free calcium concentrations of 0.1, 0.4, 1.0, 2.0 and 3.0 µM used in the experiments described, were 1.81, 4.71, 6.93, 8.22 and 8.76 mM, correspondingly.

4. Preparation of skinned muscle fibers and ghost fibers

Skinned fibers were prepared according to the method described earlier (Saks *et al*. 1998). The animals were anaesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.), chest opened and hearts when still beating excised and put into cooled solution A. Cooled hearts were cut into halves and muscle strips (3–5 mm long and 1–1.5 mm in diameter, 5–10 mg of wet weight) cut from endocardium of left ventricles along fiber orientation to avoid mechanical damage of the cells. By using sharp-ended forceps or needles, the muscle fibers were separated from each other leaving only small areas of contact. After that the fibers were transferred into vessels with ice-cold solution A containing 50 μg of saponin per ml and incubated at mild stirring for 30 min for complete solubilization of the sarcolemma. Permeabilized (skinned) fibers were then washed in solution B for 5 min; this procedure was repeated twice to remove completely all metabolites, especially trace amounts of ADP. Complete removal of ADP can be easily seen from respiration recordings which should show very reproducible initial State 2 rates (designated as v_o) not sensitive to inhibition by atractyloside.

Ghost fibers were prepared by extraction of skinned fibers in solution containing 0.8 M KCl, which resulted in residual weight of the fibers 67.6±3.5% (n = 8).

5. Isolation and culturing of adult cardiac myocytes

Isolation and culturing of adult cardiac myocytes was carried out as previously described by Kay *et al*. (Kay *et al*., 1997).

6. Isolation of rat heart mitochondria

Isolation of rat heart mitochondria was carried out as described previously (Saks *et al*., 1975).

7. Determination of the kinetics of respiration regulation by endogenous ADP (exogenous ATP) in skinned fibers and cardiomyocytes

The rates of oxygen uptake were recorded by using the two-channel high-resolution respirometer (Oroboros Oxygraph, Paar KG, Graz, Austria) or Yellow Spring Clark oxygen electrode in solution B, containing respiratory substrates and 5 mg/ml of fatty acid free BSA. Determinations were carried out at 25°C; solubility of oxygen was taken as 215 nmol per ml. Some control experiments were carried out in Ca^{2+} free sucrose medium of the following composition: 240 mM sucrose, 20 mM Hepes, pH 7.1, EGTA 1 mM, 4 mM $MgCl_2$, 3 mM KH_2PO_4, 5 mM glutamate and 2 mM malate.

8. Confocal microscopy

8.1. Imaging of autofluorescence of mitochondrial flavoproteins

Flavoproteins were imaged using a confocal microscope (LSM510 NLO, Zeiss) with a 40x water immersion lens (NA 1.2). The use of such a water immersion prevented from geometrical aberrations when observing in vitro living cells. The autofluorescence of flavoproteins was excited with the 488 nm line of an Argon laser, the laser output power was set to an average of 8 mW. The fluorescence was collected through a 510 nm dichroic beam-splitter and a 505–550 nm band pass filter. The pinhole aperture was set to one Airy disk unit.

8.2. Simultaneous imaging of flavoproteins and NADH

Autofluorescence of flavoproteins was imaged using a confocal microscope (LSM510NLO, Zeiss) with a 40x water immersion lens (NA 1.2) as described above. The autofluorescence of NADH was excited by two-photon absorption using a femto-second pulsed infra-red laser (Tsunami/MilleniaVIII, SpectraPhysics). The pulse frequency was set at 100 MHz with a pulse width of 100 femtosecond. The infra-red line was tuned to 720 nm giving a maximum two-photon absorption at 360 nm. The laser output power was set to an average power of 400 mW. The fluorescence signals were collected through a multiline beam splitter with maximum reflections at 488±10 (for rejection of the 488 nm line) and above 700 nm (for rejection of infra-red excitation). A 5 second 490 nm beam splitter was used to discriminate the NADH signal from the flavoprotein signal. Then the flavoprotein signal passed through a 500–550 nm band-pass filter with an additional infra-red rejection filter before being

collected through a pinhole (one Airy disk unit). The NADH signal was redirected to a 390–465 nm band-pass filter with an additional infra-red rejection filter.

8.3. Imaging of mitochondria in skinned cardiac fibers

The cardiac fibers were gently stirred in solution A in the presence of the mitochondria-selective dye Mito Tracker 2 Red CMXRos (Molecular Probes, Inc., Eugene, OR, U.S.A.) (200 nM) in the dark for 30 min. Thereafter the fibers were washed three times in above mentioned medium without dye for 15 min by stirring, to minimize background fluorescence. The stained fibers were incubated in HistoPrep 10% prechilled buffered 10% formalin solution (Fisher Scientific, Pittsburgh, PA, U.S.A.), containing formaldehyde (4%), methyl alcohol (1%) and phosphate buffer for 15 min. Thereafter the fibers were placed on and attached to the specimen glass with a drop of glycerol/PBS (phosphate-buffered saline) (mixed 1:1), and covered by a cover slip. All procedures were performed at 20 °C. The mitochondria were revealed and scanned using a MRC 1024 Bio-Rad laser confocal microscope with the PlanApo 60x/1.40 oil objective (Olympus, Tokyo, Japan). The specimen was illuminated by krypton/ argon laser (15 mW) light (568 nm) and the emitted light signal was filtered by a 605DF32 filter and collected according to the Kalman method using the Bio-Rad acquisition system.

8.4. Immunofluorescence confocal microscopy

For labeling of a cytoskeletal network in permeabilized fibers, monoclonal antibodies against β-tubulin were used. Cells were first washed in solution B before being fixed with methanol for 5 min at –20°C. Cardiomyocytes or fibers were washed with phosphate-buffered saline (PBS; Biomedia, Boussens, France) and incubated in 2% (w/v) BSA in PBS overnight at 4°C with primary monoclonal antitubulin antibody (Sigma) at a 1/200 dilution. After washes in PBS, cells were incubated for 3 h in 2% (w/v) PBS/BSA with secondary antibody rhodamine tetramethyl rhodamine isothiocyanate (TRITC)-conjugated AffiniPure $F(ab')_2$ fragment donkey anti-mouse IgG at a dilution of 1/50 (Interchim, Montluçon, France). Cardiomyocytes or fibers were then washed once in PBS and three times in water. The labeled cells were deposited on glass cover slips and mounted in a mixture of Mowiol® and glycerol to which 1,4-diazabicyclo-[2,2,2]-octane (Acros Organics, Pittsburgh, PA, USA) was added to delay photobleaching. Samples were observed by confocal microscopy (LSM510 NLO; Zeiss) with a plan apo 40x oil immersion objective lens (NA 1.4)

9. Electron microscopy

Muscle tissue probes were fixed in 0,25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7,4) and postfixed in 1% OsO_4 in the same buffer. After dehydration with ethanol and acetone, the specimens were embedded in Epon 812. Thin sections were stained with uranyl acetate followed by lead citrate and examined in Tecnai 10 Electron Microscope (Philips, FEI Company, Eindhoven, Netherlands) at 100 kV.

10. Fluorimetric determination of membrane potential in isolated heart. mitochondria

Rhodamine (Rh) 123 fluorescence was recorded at 503 nm (excitation)-530 nm (emission) in Photon Technology International Fluorescence Imaging System. The fluorescence intensity of Rh 123, 0.25 AM, in 2 ml of lightly stirred solution B at 25^{o}C was recorded, then isolated rat heart mitochondria were added to a final concentration of 0.1 mg/ ml. The Rh 123 uptake in response to addition of mitochondria in the presence of substrates (glutamate, 5 mM, malate 2 mM) shows the generation of a transmembrane gradient of electrochemical potential ($\Delta\psi$). The addition of an uncoupling agent, FCCP (0.1 AM), completely depolarizes the membrane due to collapse of $\Delta\psi$ and results in the release of the Rh123 out of the mitochondria and return to initial level of fluorescence. To test the stability of the system, the fluorescence of the Rh123 in the presence of energized mitochondria was recorded for more than 1 h. Usually, the level of fluorescence was stable during this period of time. In some experiments the medium contained (instead of glutamate/malate) 1 mM ATP to create $\Delta\psi$ by the reversal of the ATP synthase (F_0F_1 complex).

11. Release of cytochrome

Release of cytochrome c was measured spectrophotometrically (Appaix *et al*., 2000).

12. Protein purification

The active oligomeric forms of full-length Bax (Bax-FL) and Bax truncated of the hydrophobic C-terminal domain (Bax-DC) were purified as described earlier

(Antonsson *et al*., 2000; Montessuit *et al*., 1999). The purified proteins were stored at –80°C. Bax-FL was stored in 25 mM Hepes–NaOH, 0.2 mM DTT, 1% (v/v) octyl glucoside, 30% glycerol (v/v), pH 7.5, and Bax DC was stored in 25 mM Hepes–NaOH, 0.2 mM DTT, 30% glycerol (v/v), pH 7.5.

13. Determination of the initial rate of MgATPase reactions

The skinned fibers of rat heart were prepared as described above. The role of mitochondria in rephosphorylating ADP produced in ATPase reactions was estimated as a decrease in the flux through the external system of ATP regeneration (PK/PEP/lactate dehydrogenase (LDH)) after switching to operation of the mitochondrial oxidative phosphorylation. Approximately 3 mg skinned fibers were incubated in the spectrophotometric (Perkin-Elmer Lambda 900) cuvette containing a medium B complemented with 5 mg/ml BSA, 5 mM PEP, 20 IU/ml PK, 20 IU/ml LDH and 0.24 mM NADH at 25 °C. The medium was continuously stirred by using the magnetic stirrer operated by the Variomag® Telemodul (Labortechnic GmbH, Germany). The changes in optical density at 340 nm were measured before and after addition of 1 mM ATP as well as after subsequent additions of the respiratory substrates (10 mM glutamate and 2 mM malate) and 98 μM atractyloside. The reaction rate was estimated from the stable and linearly time-dependent portions of the recordings. Care was taken that neither of the coupled enzyme-system components was limiting the rate of ATPase reaction.

14. Determination of the steady state rate of MgATPase reactions by measuring ADP concentration in the reaction medium by HPLC

14.1. Perchloric acid extraction

200 μl of ice-cold 1 M perchloric acid were added to 100 μl aliquot withdrawn from the oxygraphic sample in order to precipitate all proteins present in the respiratory medium. The samples were further neutralized by addition of ice-cold 1 M KOH and supplemented by 0.1 mM diadenosine pentaphosphate to inhibit traces of adenylate kinase not fully denaturated in the perchloric acid medium. The samples were centrifuged at 5 000g for 3 min at room temperature and the supernatants immediately loaded into the HPLC column.

14.2. Chromatography

Determination of adenine nucleotides in chromatographic samples was performed making use of C_{18} reversed phase column (Nucleosil 5 C18 100A from Phenomenex) along with the DuPont 8800 HPLC equipment with the spectrophotometric detector at 253 nm. The column was equilibrated with 100 mM KH_2PO_4 buffer, pH 6.0, and eluted in isocratic conditions at 2 ml/min rates. Elution of ATP, ADP and AMP standards gave the retention times 2.45, 3.10 and 5.44 min, respectively. Calibrations were performed according to the peak areas of the standard mixtures of low variable amounts of ATP (0–10 μmoles) at a constant amount of ADP (100 μmoles) and vice versa.

15. Creatine concentration determination

The total Cr concentration was determined in supernatants after treatment of cardiomyocytes with saponin or Triton X-100 and centrifugation for 5 min at 1000 x g. The assay was performed by a colorimetric diacetyl a-naphthol method after hydrolysis of PCr in acidic medium and neutralization (Eggelton, 1943).

16. Lactate dehydrogenase determination

The activity of LDH in the supernatants after treatment of cardiomyocytes with saponin or Triton X–100 was assayed as described earlier (Bergmayer, 1974).

17. Trypsin treatment

The concentration of the active enzyme was determined by titration with p-guanidino-p-nitrophenyl benzoate (Chase and Shaw, 1969). The used preparation contained 48.0 ± 2.5% of active enzyme. The treatment of skinned fibers with trypsin has been performed using 50 nM enzyme for 5 minutes in solution B not supplemented with BSA, and followed by washing the preparation trice in solution B supplemented with 5 mg/ml BSA.

18. Two-dimensional electrophoresis

18.1. Sample preparation

Skinned fiber samples of 20 mg were washed in 50 mM Tris/HCl (pH 7.1)/100 mM KCl/6 mM EDTA/5.8 µM benzamidine/ 2.1 µM leupeptin for 20 s, pulverized after having been frozen with liquid nitrogen and solubilized in 100 µl of buffer which contained 9 M urea, 25 mM Tris/HCl (pH 7.1), 50 mM KCl, 3 mM EDTA, 2.9 mMbenzamidine, 70 mM DTT and 2% (v/v) ampholyte (Servalyte; pH 2–4; Serva, Heidelberg, Germany). Finally, 2 µl of additional protease inhibitors (50 mM phenylmethanesulfonylcloride) were added.

18.2. Two-dimensional gel electrophoresis and data analysis

The two-dimensional electrophoresis of proteins with carrier ampholytes and the data analysis of gels have been described previously (Jungblut *et al*., 1994; Knecht *et al*., 1994 a; b; Muller *et al*., 1996). 150 µg/l protein was loaded onto each gel. The gel size was 23 cm x 29 cm x 0.15 cm. The silver-stained gels (Knecht *et al*., 1994a) were digitized at 176 µm resolution by the use of a white light scanner (Howtek Inc., Hudson, NH, U.S.A.), and analyzed for qualitative and quantitative variations in their protein patterns by the use of the PDQUEST software package (Protein and DNA Imageware Inc., New York, NY, U.S.A.).

18.3. Analysis of the experimental results

The values in tables and figures are expressed by means ± S.E.M. The apparent Km values were determined by the non linear least squares' fit and the Vm values calculated on the base of wet weight of the fibers. Statistical comparisons were made using the Anova test (variance analysis and Fisher test), and $P<0.05$ was taken as the level of significance.

RESULTS AND DISCUSSION

1. Exogenous ADP and mitochondrial outer membrane in situ (Paper I and Paper II)

The phenomenon of high apparent Km for exogenous ADP in regulation of mitochondrial respiration compared to the isolated mitochondria has been observed in many laboratories (Table I in the Paper I). Clear distinctions in the behavior of mitochondria *in vitro* and in living cells due to intracellular protein–protein interactions and compartmentation of both the enzymes and metabolites in the cell exist. To reveal the cellular mechanism of the decreased affinity of mitochondrial respiration for exogenous ADP in muscle cells *in situ*, one needs to quantitatively evaluate the influence of diffusion of ADP from medium into the cells on the kinetics of regulation of respiration by exogenous ADP (Table 1, Paper I). In isolated cardiomyocytes the cell diameter is 16–20 μm, and thus the diffusion distance (or distance from medium into cell core) for ADP does not exceed 10 μm. The exceptions are the fast-twitch glycolytic muscles, where the average diffusion distance is much higher, about 40–50 μm but apparent Km for exogenous ADP is comparable of isolated mitochondria (8–22 μM). This clearly shows that we cannot explain the high values of apparent Km for exogenous ADP by formation of ADP concentration gradients between the medium and the core of bundles (Paper I, also Paper V).

MgATPase activity of fibers of various muscle types in the experimental conditions also do not correlate with affinity of mitochondrial respiration for exogenous ADP. Thus, the apparent Km for exogenous ADP is not dependent on the MgATPase activity (Fig. 2, Paper I).

The perforation of the outer mitochondrial membrane by the proapoptotic protein Bax reduces the apparent Km for exogenous ADP to the level close of to that for isolated mitochondria (Fig. 4, Table I, Paper II). This is in concord with the conclusion that the outer mitochondrial membrane permeability for ADP is decreased in skinned fibers.

Immunofluorescence studies and Western blot analysis showed that Bax was always present in the mitochondria of permeabilized cardiomyocytes and isolated mitochondria (from intact rat heart shown). In spite of the presence of some Bax in rat heart mitochondria, they were characterized by intact outer membrane and stable respiratory activities. Alteration of the outer membrane, and release of cytochrome c required addition of activated exogenous Bax. The effects of Bax full-length (Bax FL) and C terminal truncated Bax (Bax DC) on respiration rate, membrane potential, MgATPase activity and kinetics of regulation of respiration were studied in isolated rat heart mitochondria and permeabilized cardiomyocytes (Paper II). The results of this study show that the detailed measurements of mitochondrial activities provide new information on

the mechanisms of action of the Bcl-2 family proteins. They show that both Bax FL and Bax DC open the pores in the outer mitochondrial membrane, which are large enough to allow diffusion of cytochrome c and ADP out of and into the space between the outer membrane and the peripheral inner membrane. The new observation was that full-length Bax increases the MgATPase activity by the partial uncoupling of the inner mitochondrial membrane and inhibits some component(s) of the respiratory chain (Fig. 7 and 8; Paper II). These effects are not related to the opening of the PTP in the inner membrane. In these experiments some depolarization of the membrane in the prence of Bax might be explained by the inhibition of respiration, but this explanation could be completely excluded by the results shown in Fig. 3 E and F (Paper II). Our experiments were performed in the presence of 4 mM $MgCl_2$, a PTP blocker and we did not detect any significant mitochondria matrix swelling, loss of the transmembrane potential and breakage of the outer mitochondrial membrane. CsA (cyclosporine A) did not also show any effect, indicating that PTP opening was not involved.

Thus, our results also show a significant difference between the full-length and the C-terminal truncated Bax proteins, suggesting that in addition to its permeabilising activity on the outer membrane, the Bax FL has a specific effect on the components of the inner mitochondrial membrane. This effect is most likely linked to the C-terminal domain of Bax. These results suggested that Bax might interact and have some effect on the components of the respiratory chain. Addition of Bax FL increased the ATPase activity three times, whereas Bax DC did not show any statistically significant stimulation. Bax FL was able to partly depolarize mitochondria both in the presence of substrates and ATP.

2. Exogenous and endogenous ADP (Paper III and IV)

In these papers we described main results of the studies of the difference between kinetics of respiration regulation *in situ* by exogenous ADP endogenous ADP. In cardiac and m. soleus fibers apparent Km for exogenous ADP in regulation of respiration was equal to 300–400 μM. However, when ADP production was initiated by intracellular ATPase reactions, the ADP concentration in the medium leveled off at about 40 μM when about 70% of maximal rate of respiration was achieved.

The striking differences in kinetics of regulation of mitochondrial respiration by exogenous and endogenous ADP (Fig. 2 and 5, Paper III), show that most probably ADP produced in the intracellular ATPase reactions is directly channeled to mitochondria without significant release into the medium. This hypothesis can be tested by using an exogenous ADP-trapping PK/PEP system,

which competes with mitochondria for ADP (Paper III). Respiration rate maintained by intracellular ATPases was suppressed about 20−30% during exogenous trapping of ADP with excess PK and PEP. ADP flux via the external PK/PEP system was decreased by half by activation of mitochondrial OxPhos. Cr (20 mM) further activated the respiration in the presence of PK/PEP. The latter show that most of the endogenously generated ADP is not easily accessible for the exogenous PK and PEP, if the mitochondrial OxPhos is actively running. Since exogenous PK can occupy the intracellular bulk water phase from where the LDH has been released during permeabilization, these results mean that the endogenously generated ADP is not released into this bulk water phase. (In the case of permeabilized cells the bulk phase is a medium, in the cells *in vivo*−it is probably a cytosol.) There may be significant restriction of ADP diffusion within ICEU at the level of mitochondrial outer membrane (control of VDAC, see above) and/or in myofibrils.

To understand and analyze the dependence of the relationship between respiration rate and ADP (or ATP) concentration in the medium upon the source of this substrate,one needs to know if ADP in the intracellular bulkwater phase can equilibrate sufficiently rapidly with surrounding medium. Two approaches have been used to solve this problem (Paper IV). The first approach is to use the Einstein-Smoluchowski equation for Brownian movement in one dimension (West, 1997):

$$D=x^2/2t \qquad (6)$$

where D is diffusion coefficient and x^2 mean-square displacement for the transit time, t.

In this case, it is important to know how rapidly ADP can be exchanged between extracellular medium and intracellular bulk phase in comparison with the rate of ADP phosphorylation in mitochondria (ATP-ADP turnover rate). Simple calculations according to Eqn. 6 show that, to traverse the sarcomere space with diameter of 1 μm, ADP or ATP need only 1.5 ms. The average time of displacement of ADP or ATP between mitochondria and external medium is 38 ms (Table 1, Paper IV). To diffuse through the water from outside into the cell centre, ADP needs about 150 ms (maximum diffusion time). These diffusion times should be compared with the time needed to rephosphorylate ADP into ATP in mitochondria (turnover time). This time is between 100–200 ms at 20°C. Thus ADP diffuses more rapidly in the space occupied by the cardiomyocytes than it is utilized, provided that its diffusion coefficient is that found is aqueous solution (Table 1, Paper IV).

The second approach is to use the mathematical reaction-diffusion model of energy transfer to study the influence of diffusion on the profile of metabolites within the sarcomere space. This was done recently (Vendelin *et al*., 2000) and the results showed that, even if the diffusion coefficient for ADP was increased

10^5 times from its value in water, the ADP profile did not change. Thus diffusion of ADP in the bulk-water phase in cells is already very rapid.

It is concluded that in oxidative muscle cells mitochondria behave as if they were incorporated into functional complexes with adjacent ADP producing systems — with the MgATPases in myofibrils and Ca,MgATPases of sarcoplasmic reticulum. Within these functional units the energy is transferred mostly via enzyme networks composed of the CK and adenylate kinase systems. These functional units between mitochondria and Mg-ATPases may be called ICEUs (Scheme I in Paper IV) and they seem to serve as a basic pattern of functional organization of the whole cell energetics.

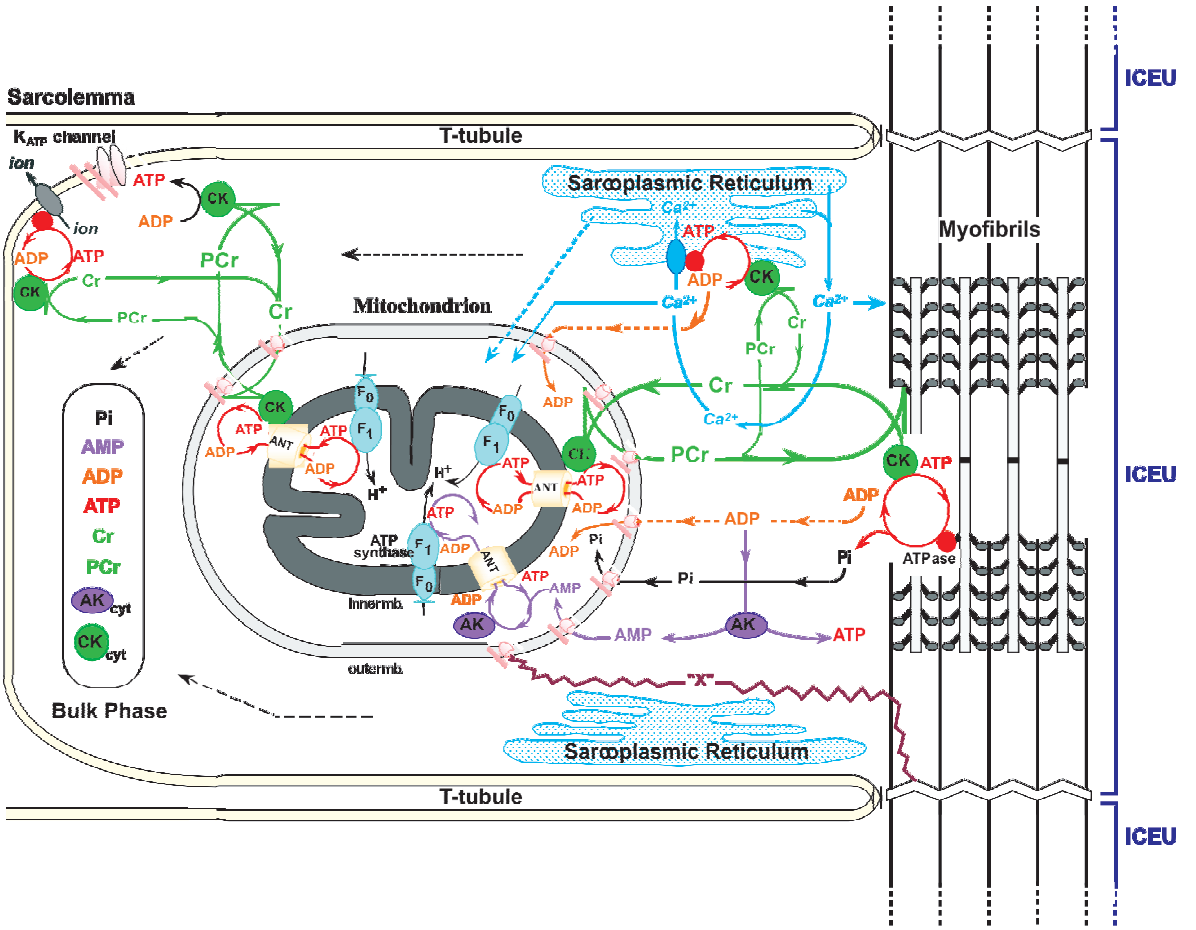
Rapid increase of the apparent affinities for exogenous ADP (Fig. 3, Paper IV) and the increase in accessibility of the endogenous ADP for the PK/PEP system after short-time selective proteolytic treatment of skinned fibers (Fig. 5, Paper IV) are observed. Electron-microscopic observations showed detachment of mitochondria and disordering of the regular structure of the sarcomere after trypsin treatment (Fig. 9, Paper IV). Two — dimensional electrophoresis revealed a group of at least seven low-molecular-mass proteins in cardiac skinned fibers which were very sensitive to trypsin and not present in glycolytic fibers, which have low apparent Km for exogenous ADP. This specific structure, ICEU, falls apart after short, probably selective, proteolysis of some structural proteins (Fig. 9 in Paper IV), while the activities of MgATPase are not changed by this treatment (Fig. 7 in Paper IV). This explains empirical observation that treatment of muscle homogenates with proteases is a necessary step in the isolation of intact mitochondria. However, the existence of ICEUs may not be limited to the striated muscle cells.

The concept of the ICEU developed in the present paper helps to explain two very important phenomena whose mechanisms are still unclear like the mechanism of cardiac-contraction failure under conditions of inhibition of OxPhos, such as ischemia or hypoxia, when the cellular content of ATP remains still high.

3. The effect of cytoskeleton in regulation of ATP production in mitochondria (Paper V and Paper VI)

The cytoskeletal network is important for mitochondrial arrangement and regulation in muscle cells in situ:

1) The differences in the kinetics of the regulation of mitochondrial respiration in cardiac cells *in vivo* and *in vitro* is tissue specific and observed in oxidative slow-twitch skeletal muscle cells but not in fast-twitch skeletal muscle cells.



Scheme I (Paper IV): Schematic presentation of functional ICEUs in the muscle cells
By interaction with cytoskeletal elements, the mitochondria and SR are precisely fixed with respect to the structure of sarcomere of myofbrils between two Z-lines and correspondingly between two T-tubules. Ca^{2+} is released from the SR into the space of the ICEU in the vicinity of the mitochondria and sarcomeres to activate contraction and mitochondrial dehydrogenases. Adenine nucleotides within the ICEU do not equilibrate rapidly with adenine nucleotides in the bulk-water phase. The mitochondria, SR and Mg-ATPase of myofibrils are interconnected by metabolic channeling of reaction intermediates and energy transfer within the ICEU by the CK- PCr- and adenylate kinase systems. The protein factors (still unknown and marked as "X"), most probably connected to cytoskeleton, fix the position of mitochondria and probably also control the permeability of the VDAC channels for ADP and ATP. Adenine nucleotides within the ICEU and bulkwater phase may be connected by some more rapidly diffusing metabolites as Cr and PCr. Synchronization of functioning of ICEUs within the cell may occur by the same metabolites (for example, Pi or PCr) and/or synchronized release of Ca^{2+} during the excitation-contraction coupling process. Abbreviation: AK, adenylate kinase (with permission).

2) Hypoosmotic shock, by disrupting outer mitochondrial membrane, decreases the apparent Km for exogenous ADP (the same effect is in chronic ischemia). This shows that the integrity of the mitochondrial outer membrane is involved in the phenomenon.
3) The kinetics of the regulation of mitochondrial respiration in situ is very different for exogenous and endogenous ADP.
4) The decrease of apparent Km during the selective proteolytic treatment, points to the participation of some cytoskeleton — related proteins, which control the permeability of the mitochondrial outer membrane.

A very regular arrangement of mitochondria can be seen between myofibrils in the skinned cardiac fibers, in good agreement with many earlier observations. This regular arrangement is rapidly disorganized by the treatment of fibers for 5 min at 4 °C with 0.1–5 μM trypsin (Fig. 5B and C, Paper V). Fig. 6 (Paper V) shows that, in parallel, the apparent Km for ADP progressively decreased to very low values of around 40–50 μM. At a very low trypsin concentration (100 nM), two or more different populations of mitochondria, and at higher trypsin concentrations, only one homogeneous population of mitochondria with very low apparent Km for exogenous ADP was seen (Fig. 6). Figure 7 (Paper V) shows that the proteolytic treatment and disorganization of the intracellular arrangement of mitochondria significantly increases the accessibility of the endogenous ADP to the added trapping PEP/PK system.

All these results show that the intracellular regular arrangement of mitochondria, high apparent Km for exogenous ADP and channeling of endogenous ADP to mitochondria are most probably related phenomena due to the presence of proteins sensitive to trypsin. The most intriguing question is: which of the cytoskeletal components might be responsible for mitochondrial arrangement and regulation?

To address this problem, the confocal microscopy was used to study changes in intracellular distribution of mitochondria and localization of cytoskeletal proteins, such as desmin, tubulin and plectin in permeabilized cardiac cells during short proteolytic treatment (Paper V). The results show rapid collapse of microtubular and plectin networks but not of desmin localization under these conditions. A parallel decrease of the apparent Km and disappearance of immunolabelling of the microtubular network may suggest the possible role of microtubules in the control of mitochondrial function (Fig. 8 in Paper V). Fig. 8A shows that the microtubular network is extremely dense and intact in the cardiomyocytes even if the cells are permeabilized and kept in solution B at 4 °C before staining. Fig. 8B shows double labeling of the microtubular network in cardiomyocytes and mitochondria: it seems that the mitochondria are wrapped into the network. Fig. 8C shows that after selective treatment by trypsin, which disorganizes the regular arrangement of mitochondria (Fig. 5), the microtubular network progressively disappears with increasing concentration of proteinase. Figure 2 (Paper VI) shows that the permeabilization

process is complete and allows excellent immunofluorescence staining of the microtubular network in the cells. The network is intact, showing that the cytoskeleton of the cells is well preserved during the permeabilization procedure.

The second protein studied is the main cytoskeletal protein in cardiac and skeletal muscle, desmin, which is expressed in all striated and smooth muscle tissues. Desmin was not significantly affected by trypsin treatment and may not be direct candidate for the control of mitochondrial function (Fig. 9 in Paper V). This is in agreement with our observations on desmin-deficient mice, where we still observed a population of mitochondria with a high apparent *K*m for ADP, and another with a very low apparent *K*m, obviously because of mitochondrial clustering as a result of a weakening of cell structure in the absence of desmin (Kay *et al.,* 1997).

Plectin may have a central role in the structural and functional organization of the intermediate filament cytoskeleton in skeletal muscle. Plectin co-localizes with desmin but trypsin treatment has not the same effect on both proteins. Indeed, desmin seems not to be affected by the proteinase whereas plectin was cleaved and its staining disappeared after treatment with 1 mM trypsin (Fig. 10). Therefore, it is not clear yet if only the plectin and microtubular network, or mostly other cytolinker proteins are important for the arrangement and control of mitochondria.

The results show that the structural differences are paralleled by differences in functional characteristics, in particular in the apparent Km for exogenous ADP. Tissue specificity seems to be true for mitochondrial-cytoskeletal interactions in general. In some cases, the molecular bases behind the organellar movement of microtubules are motor proteins, kinesin and cytoplasmic dynein, which bind microtubules and transduce chemical energy of ATP into mechanical work of mitochondrial movement along microtubules (Yaffe, 1999). One may think that in cardiac cells the mitochondria have arrived at their proper, fixed position inside functional complexes with sarcomeres and SR (ICEUs) to achieve the most effective regulation of cellular energetic. Indeed, during cardiac muscle development, intracellular distribution of mitochondria changes from a chaotic one in the early postnatal period to a very regular arrangement in the adult muscle (Tiivel *et al*., 2000). Since interaction with cytoskeleton is mediated by proteins that are associated with the outer mitochondrial membrane (Leterrier *et al*., 1994; Smirnova *et al*., 1998; Yaffe, 1999), it is easily feasible that these proteins also control the permeability of the voltage-dependent anion (VDAC) channels of the outer mitochondrial membrane (Colombini, 1994) to adenine nucleotides. However, while the collapse of the microtubular network (Paper V) during short proteolysis coincides with disorganization of the regular arrangement of mitochondria in cardiac cells and an increase in the apparent affinity for exogenous ADP in regulation of respiration as a result of elimination of local restrictions of diffusion, it is not clear if only the microtubular network participates in distribution of

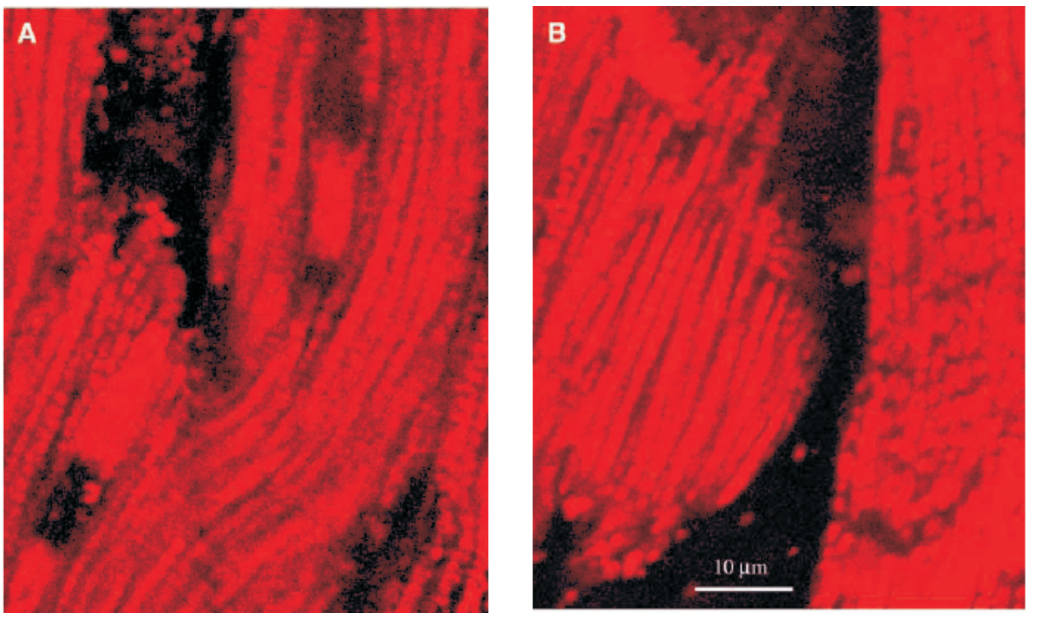


Figure 6 (Paper VI). Imaging of mitochondria in permeabilized myocardial fibers by the membrane-potential-sensitive probe teramethylrhodamine ethyl ether. (A) Fibers in the presence of 2·mM· ATP, 2·mM ·malate and 5·mM· glutamate (concentration of free Ca^{2+} in Ca-EGTA buffer: 0.1·mM). (B) The same fibers after addition of calcium chloride (final concentration of free Ca^{2+} in Ca-EGTA buffer 1.0·mM). The left fiber in a flexiperm chamber was not fixed, while the right (longer) fiber was fixed by its ends. In A, both fibers are relaxed. In B, the left fiber is contracted, while the right fiber, which is contracting almost isometrically, shows significant structural changes due to sarcomere contraction. Note the empty spaces between mitochondria in this case (with permission).

mitochondria in the cells and which type of cytolinker proteins is associated with the mitochondrial surface to fix them precisely inside the cells as discussed above.

4. The effect of Ca^{2+} on the mitochondrial respiration in situ (Paper VI)

An increase in the free Ca^{2+} concentration (up to 3 μM, which is within physiological range) resulted in a very significant decrease of the apparent Km value to 20–30 μM, a slight decrease of Vmax of respiration in permeabilized intact fibers and a strong contraction of sarcomeres. Fig. 8 (Paper VI) shows the apparent link between sarcomere length and the affinity of mitochondria for exogenous ADP measured as an apparent Km. In the presence of ATP (or respiratory substrates and ADP), an increase of free Ca^{2+} concentration to 3 μM results in strong contraction of sarcomeres and shortening of fiber length in intact permeabilized cardiac fibers. If the fibers are not fixed, intermyofibrillar mitochondria seem to fuse as a result of being pressed together; if the fibers are fixed in flexiperm and contract isometrically (Fig. 6, Paper IV), one observes the appearance of the empty areas and of a rather long distances between mitochondria. In the cases of high Ca^{2+} concentrations, the structure of the cell and the structure of ICEUs are deformed. Structural connections between mitochondria and sarcomeres (and probably SR) inside ICEUs are so strong that there exists a direct link between sarcomere length and regulation of mitochondrial function. In ghost cardiac fibers, from which myosin was extracted but mitochondria were intact, neither the high apparent Km for ADP (300–350 mmol/l) nor Vmax of respiration changed in the range of free Ca^{2+} concentration studied. In these ghost fibers, the regular distance between mitochondria, corresponding to sarcomere length, is not changed with alteration of calcium concentration (Fig. 7). Thus, there is no deformation of the internal, modified structure of the ICEUs in the cell. Stability of all mitochondrial functions in ghost fibers shows that changes in the free Ca^{2+} concentration in the range used does not alter the mitochondria, which might result from a mechanism of the permeability transition pore (PTP) opening.

The strong effect of sarcomere contraction on the apparent Km for exogenous ADP observed in this work (Fig. 8, Paper IV) shows that structural connections between mitochondria and sarcomeres inside ICEUs are very significant. One of the possible explanations of this surprising phenomenon is that sarcomere contraction results in deformation of the mitochondrial outer membrane and opening of the VDAC pores to adenine nucleotides. Another possibility is that significant shortening of sarcomere length changes the structure of the ICEUs in general and makes the diffusion of exogenous ADP to mitochondria inside the cells easier.

5. Heterogeneity of intracellular ADP diffusion as the main function of ICEU (Paper VII)

Structural organization of mitochondria into functional complexes with myofibrils and SR (ICEUs) may be a basic pattern of organization of energy metabolism in the oxidative muscle cells and may lead to heterogeneity of ADP diffusion in the cells.

Heterogeneity of ADP diffusion and regulation of respiration were studied in permeabilized cardiomyocytes and cardiac fibers *in situ* and *in silico*.

Application of confocal imaging of mitochondria revealed two important aspects. First, very high degree of order in the mitochondrial distribution and arrangement in the cardiac cells was demonstrated. Mitochondria in unfixed muscle fibers were found to localize in parallel rows between myofibrils and positioned in the middle of sarcomeres at the level of A-band, giving rise to the striated pattern of their intracellular arrangement in muscle cells visible from confocal images (Fig. 1). The second observation is that this type of organization, these structural and functional complexes of mitochondria can be extremely easily destroyed by short proteolytic treatment (Fig. 4B), this resulting in loss of the heterogeneity of ADP diffusion.

Channeling of endogenous ADP to mitochondria within these complexes was studied by using the PK/PEP system (Gellerich and Saks, 1982). The effects of PK/PEP on mitochondrial respiration in permeabilized fibers activated by endogenous ADP in the presence of 2 mM MgATP were studied before and after treatment with 1 mM trypsin. Oxygraphic measurements have shown that neither the intactness of the mitochondrial outer membrane as measured by cytochrome-c test nor the maximal capacities of respiration per wet weight the respiratory parameters were changed by trypsin treatment experiments. In intact fibers, the increase of PK activity to 20 IU/ml decreased the rate of oxygen consumption to not more than 30–40% (Fig. 4C). Activation of CK reaction by Cr in the presence of MgATP significantly increased the respiration rate up to 120–130% of the initial value, which is close to the maximal State 3 respiration rate (Fig. 4 C). Remarkably, this effect was seen despite the presence of the PK/PEP system. This result is explained by local production of ADP in the intermembrane space by the MitCK reaction functionally coupled to the ANT. The degree of inhibition of respiration by PK-PEP system in the absence of Cr was drastically (80%) increased by trypsin treatment (Fig. 4, Paper VII, Fig. 24). In this case most of the endogenous ADP became available for the PK/PEP system before being used by mitochondria. Cr still activated the respiration, but to a remarkably lesser extent than in the control (Fig. 4, Paper VII), most probably because of the increased permeability of the outer mitochondrial membrane for ADP after trypsin treatment (Fig. 24). Under these conditions, it is known that 20 mM Cr activates the MitCK

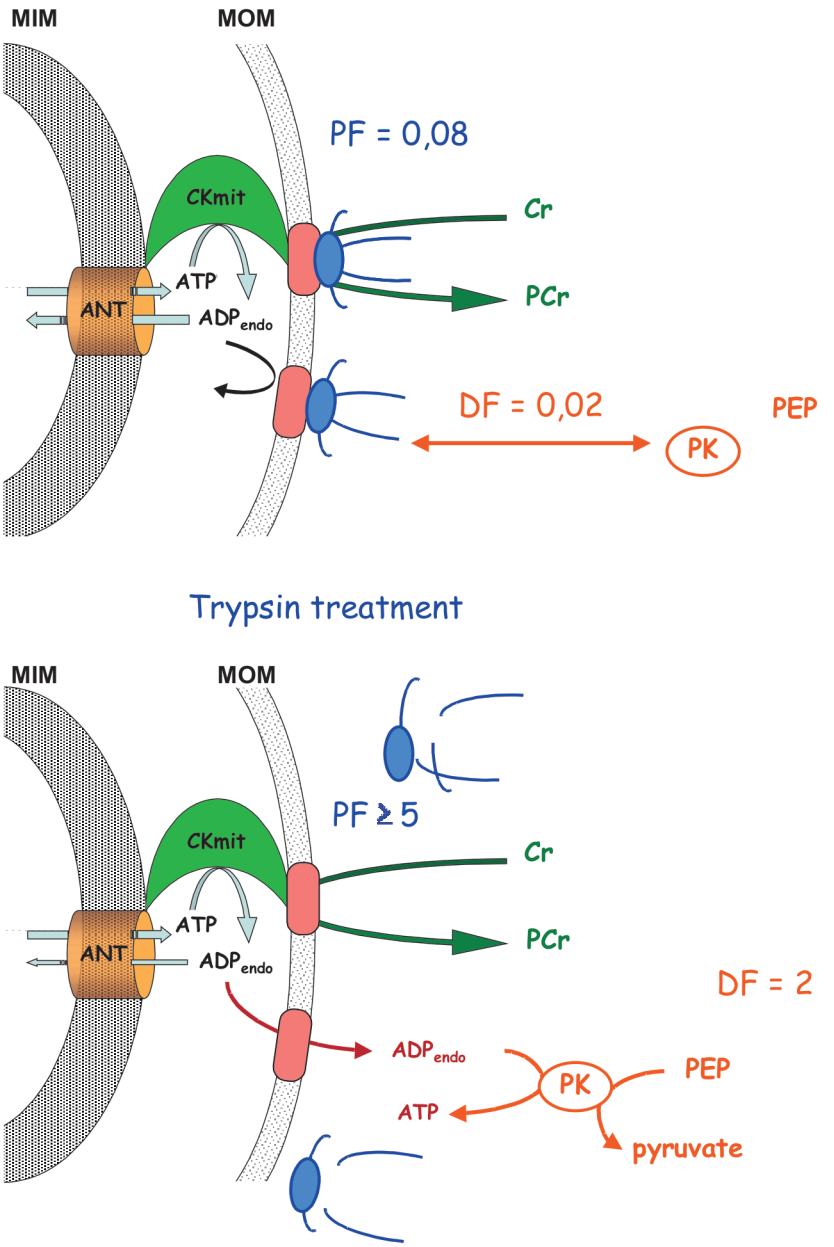


Figure 24. Schematic presentation of the effect of trypsin treatment. Decrease of the activation of respiration by Cr after disorganization of regular arrangement of mitochondria by trypsin treatment shows increased leak of ADP from the intermembrane space through outer membrane, and thus the increased permeability of the latter. In trypsinized fibers, mitochondrial outer membrane becomes permeable for ADP as in isolated mitochondria in vitro. (MIM?mitochondrial inner membrane, MOM–mitochondrial outer membrane, DF–diffusion fraction, PF–permeability fraction, PF, describing the permeability of the mitochondrial outer membrane for ADP, See also Appendix). (From T. Andrienko, personal communications).

maximally, and the functional coupling of MitCK with ANT is intact in mitochondria isolated with the use of trypsin (Saks *et al*., 1975). After disorganization of ICEUs by trypsin, Cr activates the respiration rate in the presence of the PK/PEP system exactly to the same extent as in experiments with isolated rat heart mitochondria under similar conditions-up to the 50% of the State 3 rate of respiration (Gellerich and Saks, 1982).

The role of mitochondrial matrix volume changes in regulation of the mitochondrial affinity for exogenous ADP was studied by changing the osmolarity and composition of solutions. Isolated cardiac mitochondria were incubated in the sucrose medium without K^+, and in solution B with 126 mM K^+. The matrix volume increases significantly because of K^+ entry via the potassium channel, which is opened in the absence of ATP (Kowaltowski *et al*., 2001). Reversal of K^+ uptake by nigericine (Nicholls and Ferguson, 2002) diminished the swelling of mitochondrial matrix (Fig. 2). The matrix volume changes during transition mitochondria from sucrose to solution B (swelling of the matrix without rupture of the membranes) had no influence on the value of the apparent Km (ADP), which remained always very low, in the range of 5–10 μM (Fig. 2). The high apparent Km for exogenous ADP in permeabilized cardiac fibers was not changed by increasing osmolarity of the solution from isoosmotic (0.24 osM) up to highly hyperosmotic conditions (1.3 osM) by addition of sucrose, the only result is a decrease of Vmax of respiration (Fig. 3). Significant decrease of osmolarity resulted in the rupture of outer mitochondrial membrane and strong decrease in apparent Km value for exogenous ADP.

The results showed that experimental data obtained in this and earlier studies can be explained by heterogeneity of diffusion of phosphorus metabolites discovered by Kinsey and de Graaf (Kinsey *et al*., 1999; de Graaf *et al*., 2000). The features of experimental dependencies of the Vo_2 on the exogenous ADP (and ATP) concentrations, including the high apparent Km for this substrate, the alterations of the apparent affinity for exogenous ADP by trypsin as well as changes of metabolic channeling of ADP by this proteolytic treatment-all could be explained by variations in the apparent diffusion constants, D^{app}, and outer mitochondrial membrane permeability, and thus by the alterations of the heterogeneity of the intracellular diffusion of ADP (and ATP).

The modified mathematical model of compartmentalized energy transfer was adapted in this work to study the diffusion of exogenous and endogenous ADP in skinned cardiac fibers before and after the disorganization of the ICEUs' structure. Quantitative analysis of the experimental data by the mathematical modeling of compartmentalized energy transfer showed that ADP diffusion inside the cells is heterogeneous and the diffusion coefficient may be decreased locally by more than an order of magnitude. This occurs in addition to restriction of permeability of the mitochondrial outer membrane for ADP in the cell *in vivo*. The quantitative analysis of these data by the model of compartmentalized energy transfer (Fig. 9 and 10) confirmed that the permeability of

the outer mitochondrial membrane is restricted in the cells *in situ* and increased after proteolytic treatment. This is clear from Fig. 9 in this paper.

Modeling the effects of Cr on the mitochondrial endogenous ADP-dependent respiration in the presence of the ADP-trapping system PK/PEP supported both the conclusion of the central role of the MitCK in regulation of respiration, and the importance of changes in outer mitochondrial membrane permeability for adenine nucleotides after treatment of fibers with trypsin. In the model, the functional coupling of MitCK with ANT was described by a phenomenological kinetic equation, reflecting metabolic channeling of ATP and ADP between these two proteins. The local pools of ADP generated by the MiCK reaction near the ANT were completely protected from the competitive PK/PEP system, despite some leaks of ADP into the intermembrane space

Appendix:
Mathemical modeling of diffusion restrictions performed by M. Vendelin

The model was used to calculate the mitochondrial respiration rates as function of: 1) the concentration of exogenous ADP; 2) the concentration of exogenous ATP as a source of endogenous ADP and 3) to analyze different effects of the PK/PEP system on the respiration before and after treatment of the fibers by trypsin. In the model, two parameters describing ADP diffusion were varied to fit the experimental data: DF, called the diffusion fraction, and permeability fraction, PF, describing the permeability of the mitochondrial outer membrane for ADP. In this model, the reaction rates of all enzymes were reduced four times in comparison with the data of earlier publication (Vendelin *et al*., 2000) to take into account the difference in the temperature (25°C was used in this study with skinned cardiac fibers instead of 37°C, used previously). The ATPase activity, V_{ATP} in skinned fibers is taken to be nonperiodic (noncontracting fibers), but stationary and dependent on the concentrations of MgATP and MgADP. Since inorganic phosphate concentration was not changed in this work but kept constant at 3 mM. The diffusion between the fiber and solution is simulated only in one cross section. The cross section was populated with mitochondria (diameter 1 μm), which were distributed randomly in the cross section to fill one-quarter of the fiber volume (Fig. A1; Paper VIII, Appendix) in accordance with confocal imaging of mitochondrial flavoproteins (Fig. 1, B and D; Paper VIII, Appendix). The diffusion path of a metabolite was divided into the following three parts (Fig. A2, Appendix): 1) restricted diffusion from solution through the cytoplasmic and myofibrillar space into the vicinity of each mitochondria, with an apparent diffusion coefficient D^{app}; 2) passive diffusion through the outer mitochondrial membrane with permeability

coefficient PF x R; 3) carrier-mediated exchanges from intermembrane space into mitochondrial matrix. The concentrations of metabolites within distinct mitochondria are different because of variations of concentrations of metabolites surrounding every mitochondria in myofibrillar and myoplasmic compartments.

The concentrations of the metabolites in mitochondrial matrix were calculated from their concentrations in the intermembrane space and by the kinetics of adenine nucleotide and phosphate transport kinetics (Vendelin *et al*., 2000). Respiration rates were calculated as the functions of the metabolite concentrations in mitochondrial matrix (Vendelin *et al*., 2000). Since the concentrations within each mitochondrion were different, the respiration rates were different too. When comparing with the experimental measurements, an average respiration rate was used.

In this model, similarly to Aliev and Saks (1997) and Vendelin and coworkers (Vendelin *et al*., 2000), functional coupling of MiCK and ANT was assumed to occur by means of high local ATP and ADP concentrations in a narrow space microcompartment ("gap") between coupled enzymes. Basic principles underlying the model composition were as in Vendelin's study (Vendelin *et al*., 2000): 1) ANT was assumed to translocate adenine nucleotides between matrix space and microcompartment and, partly, intermembrane space; 2) MitCK was linked to ATP and ADP both in the intermembrane space and microcompartment; 3) Diffusion between the microcompartment and intermembrane space was considered to be restricted; 4) Due to the infinitely small capacity of the microcompartment, for each adenine nucleotide, the influx was taken to be equal to its efflux.

The new model of MitCK/ANT coupling is a simplified version of the approach presented earlier (Vendelin *et al*., 2000), involving isolated mitochondria with ANT and MitCK bound to the inner membrane. The adequacy of the model was tested to the experimental data obtained from measurements with isolated mitochondria: 1) Dependence of the rate of PCr production on ATP concentration was measured at different Cr and PCr concentrations in the presence of OxPhos (Jacobus and Saks, 1982); 2) After adding PK into the solution containing respiring mitochondria and PEP, the decrease in the rate of OxPhos. as a function of PK activity was detected (Gellerich and Saks, 1982). The relative activities of MitCK and PK were estimated from the measured rate of OxPhos assuming that the MitCK reaction rate is equal to the rate of ATP production in mitochondrial OxPhos (Jacobus and Saks, 1982); 3) in isolated mitochondria, the CK reaction coupled to OxPhos results in the reversal of the CK reaction as opposed to the noncompartmentalized CK in case of nonrespiring mitochondria in certain conditions (Saks *et al*., 1975).

To analyze the problems of restriction of diffusion in some regions inside the cells, first the diffusion coefficient for metabolites inside the control fibers (D^{app}) was taken to be equal to its value in the bulk water phase of cells (D_0, DF=1), but the permeability of the mitochondrial outer membrane for ADP

(expressed as PF) was varied, in accordance with our initial hypothesis (Saks *et al*., 1995). The comparison of these results with experimental data on the dependence of the rate of respiration on the exogenous ADP concentration (Fig. 5, Paper VII) showed that under these conditions, the increase of the outer membrane permeability for ADP increases the affinity of the system for ADP, as expected, approaching the experimental data for isolated mitochondria in vitro. The value DF=1 does not explain the effect of trypsin on the competitive inhibition of endogenous-ADP dependent respiration by the PK/PEP system, there must be some additional restrictions for ADP diffusion outside the outer mitochondrial membrane; for example, between different functional units. The procedure to find optimal DF is shown in Fig. 6, A and C (Paper VII), and PF simultaneously for best fitting in Fig. 6, B and D. The optimal values of DF are in the range between 0.02 and 1 (; Fig. 6 and Fig. 7A, Paper VII) and PF in the range between 0.02 and 0.08 (Fig. 6B and D, Paper VII). That means that the average apparent diffusion coefficient for ADP may be decreased by one or even two orders of magnitude somewhere between the functional complexes (ICEUs) and the medium, and the permeability of the outer mitochondrial membrane for ADP may also decreased in comparison with that for isolated mitochondria.

The efficiency of the PK/PEP system in competition with mitochondria for endogenous ADP depends on the rates of diffusion of endogenous ADP inside the fibers between Ca,MgATPases and mitochondria, and between Ca,MgATPases and solution. For nontreated fibers good fit between calculated and experimental data is found for the values of DF smaller than 0.06, the best fit for DF=0.02. When the diffusion coefficient for ADP was that for the cellular bulk water phase (DF=1), the calculated effect of the PK system was very strong and the theoretical curves in Fig. 8, A and C (Paper VII) are far below the experimental points for intact fibers. After treatment with trypsin, when the structure of muscle cell was disorganized, in the presence of PK the good fit was achieved for DF=2 (Fig. 8B and D, Paper VII): theoretical curve was within the range of experimental errors. Some restriction of diffusion may still be present, however, since the mean experimental values are slightly higher for lower PK activity than the theoretical curve (Fig. 8).

Important information on the distribution of diffusion restrictions in the permeabilized cardiac cells can be obtained from the stimulation of respiration by addition of 20 mM Cr in the presence of the PK/PEP system. In all cases the stimulatory effect of Cr on the respiration in the presence of the powerful ADP-consuming PK/PEP system is well reproduced by the model, and the degree of stimulation remarkably depends upon the values of the PF for mitochondrial outer membrane. For nontreated fibers, the computed respiration rate in the presence of creatine was always within the experimental error range, with slight decrease of VO_2 with increase of DF (Fig. 8A and C, Fig. 9, Paper VII),

The influence of DF and PF on the rate of respiration stimulated by Cr is analyzed quantitatively in Fig. 9. Simulated rates of respiration always exceed

the initial one, and decrease of DF resulted in increase of PF and vice versa (Fig. 9A and C, Paper VII). After treatment with trypsin, when DF was taken to be equal to 2, the only parameter of importance was PF, good fit of mean experimental value with simulation results was observed for PF exceeding 5. That means that treatment with trypsin results in significant increase of permeability of the mitochondrial outer membrane.

Finally, we simulated directly the dependence of VO_2 on the exogenous ADP concentration after the treatment of fibers with trypsin (Fig. 10 A). The usually observed apparent Km values equal to 300–350 μM correspond to DF values; 0.06 and corresponding PF values; 0.03 (Fig. 10 B). When the cell structure is disorganized by trypsin and DF = 2, increase in PF results in rapid decrease of the apparent Km for exogenous ADP. In this case the experimental values of the apparent Km for exogenous ADP between 40 and 70 mM correspond to the range of PF values >0.5–1 (Fig. 10 C).

The values of PF may be decreased in the permeabilized cells in situ up to two orders of magnitude, and that of DF by one order of magnitude, in comparison with the isolated mitochondria in vitro.

CONCLUSIONS

1) The high values of K_m^{app} for exogenous ADP in regulation of mitochondrial respiration observed in skinned fibers of oxidative muscles cannot be explained by formation of large concentration gradients between cell interior and medium along which ADP diffuses with fixed diffusion constant as in water solution. On the contrary, the high Km values for exogenous ADP are related to limited diffusion (local restriction of diffusion and decrease of diffusion coefficient value) inside the cells due to high degree of structural organization. High apparent Km for exogenous ADP points to existence of the structurally organized feedback signaling system in the cells.
2) The proapoptotic protein, Bax-FL, affects both the outer and the inner mitochondrial membranes; by forming large pores in the outer membrane without opening the permeability transition pore it decreases significantly the value of apparent Km for ADP in regulation of respiration. At the leve of the inner membrane, Bax FL inhibits some segments of the respiratory chain and increases the proton leak.
3) Mitochondrial respiration is dependent on the source of activator ADP: endogenous ADP generated by ATPases has much higher access to mitochondria than ADP provided externally. Therefore, it appears that in oxidative muscle cells mitochondria behave as if they were incorporated into functional complexes with adjacent ADP producing systems – with the MgATPases in myofibrils and Ca,MgATPases of sarcoplasmic reticulum – the intracellular energetic units (ICEUs). Organization of mitochondria into ICEUs results in the heterogeneity of the intracellular diffusion of ADP and ATP.
4) The ADP diffusion is restricted between and inside the ICEUs, as suggested by small inhibitory effects of the exogenous ADP trapping PK/PEP system on oxidative phosphorylation in permeabilized cells. Probably ADP diffusion is restricted at the level of outer mitochondrial membrane due to the control of porin pores by some cytoplasmic proteins.
5) The collapse of microtubular network during short proteolysis coincides with disorganization of the regular arrangement of mitochondria in cardiac cells and an increase in the apparent affinity for exogenous ADP. Plectin or mostly other cytolinker proteins are important for the arrangement and control of mitochondria.

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SUMMARY IN ESTONIAN

Rakusisene energeetilin ühik: struktuursed ja funktsionaalsed aspektid

Dissertatsiooniga seotud töö tulemusena on püstitatud rakusisese energeetilise üksuse (EÜ) hüpotees, mille kohaselt südamelihases ja aeglastes skeletilihastes aktomüosiinisüsteem, mitokondrid ja sarkoplasmaatiline retiikulum moodustavad ühtse funktsionaalse terviku, milles ATPaaside poolt produtseeritav ADP suunatakse otse mitokondritesse ATP resünteesiks ilma ADP vaba difusioonita tsütoplasmasse. EÜ- siseselt fikseerivad ja reguleerivad mitokondreid tsütoskeleti elemendid; tsütoskeleti valkude limiteeritud proteolüüsil taoline regulatsioon kaob. Seda hüpoteesi uuriti detailselt rakendades konfokaalmikroskoopiat, 2D-elektroforeesi ja ensüümreaktsioonide kineetilise analüüsi meetodeid. Töö käigus märgistati ka erinevaid tsütoskeleti valke (tubuliin, plektiin, desmiin) ning uuriti proteaaside toimel lihasrakus toimuvaid funktsionaalseid ja struktuurseid muutusi. EÜ funktsionaalseid omadusi muudeti varieerides vaba [Ca^{2+}] füsioloogilistes piirides (0–3μM) ning registreeriti kaasnevaid hingamisparameetrite (Km ja Vm) muutusi skineeritud lihaskiudes ja nende "varjudes", milles põhiline osa müosiini ATPaasist on eemaldatud. Mitokondrite välismembraani rolli hingamise regulatsioonis uuriti kasutades selle membraani läbitavust moduleeriva proapoptootilise valgu Bax erinevaid vorme.

Töö tulemused võimaldasid teha järgmisi järeldusi:

1) Mitokondriaalse hingamise regulatsioonis registreeritud näilise Km kõrged väärtused ADP suhtes oksüdatiivsete lihaste skineeritud kiududes ei ole seletatavad raku sise- ja väliskeskkonna vahel kujunevate suurte ADP difusioonigradientidega, mille toimel ADP diffundeeruks rakku vesikeskkonnas toimiva ja fikseeritud difusioonikonstandi juures. Sellise mehhanismi asemel põhjustab kõrge Km väärtuse eksogeense ADP difusiooni piirang lokaalse difusioonitakistuse ja sellest tuleneva difusioonikontandi vähenemise tõttu rakus, tingituna raku kõrgest struktureerituseastmest. Kõrge näiline Km eksogeense ADP suhtes viitab struktuurselt organiseeritud ja tagasisidestatud signaalsüsteemi eksisteerimisele südamelihase rakkudes.
2) Proapoptootiline valk Bax-FL mõjutab nii välist kui sisemist mitokondri membraani; moodustades uuri poore välismembraanis, kuid indutseerimata megakanalit, vähendab ta näilist Km ADP suhtes hingamise regulatsioonis. Mitokondri sisemembraanis inhibeerib Bax-FL hingamisahela mõningaid segmente ja suurendab prootonleket.

3) Mitokondrite hingamine sõltub aktiveeriva ADP allikast: ATPaaside poolt genereeritud endogeenne ADP on palju paremini kättesaadav mitokondritele kui väliselt lisatud ADP. Seetõttu näib, et oksüdatiivsetes lihasrakkudes käituvad mitokondrid nii nagu moodustaksid nad funktsionaalseid komplekse külgnevate ADP-d produtseerivate süsteemidega — Mg-ATPaasidega müofibrillides ja Ca,Mg-ATPaasidega sarkoplasmaatilises retiikulumis, ehk rakusiseseid energeetilisi üksuseid (EÜ). Mitokondrite ja ATPaaside organisatsioon EÜ-desse põhjustabki ADP ja ATP rakusisese difusiooni heterogeensuse.
4) ADP difusioon takistub EÜ-de vahel ja sees, millele viitab eksogeenset ADP-d siduva PK/PEP süsteemi vähene inhibeeriv toime oksüdatiivsele fosforüülimisele permeabiliseeritud rakkudes. Võimalik, et ADP difusioon takistub mitokondri välismembraani tasemel mõningate tsütoplasmaatiliste valkude poriinikanaleid kontrolliva toime tõttu.
5) Mikrotubulaarse võrgustiku purunemine limiteeritud proteolüüsi toimel viib mitokondrite regulaarse paigutuse kadumisele kardiomüotsüütides ning mitokondrite näilise afiinsuse kasvule eksogeense ADP suhtes. Plektiin ja võimalik et ka mõned teisted tsütolinkervalkud osalevad mitokondrite funktsiooni ja asendi määramisel südamelihases.

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PUBLICATIONS

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Teadustegevus

Peamisteks uurimisvaldkondadeks on südame ja skeletilihaste hingamise regulatsiooni uurimine *in vivo* tingimustes ning ning mitokondrite ja tsütoskeleti vaheliste interaktsioonide funktsionaalse tähtsuse selgitamine. Ilmunud on 14 teaduspublikatsiooni rahvusvahelistes ajakirjades.

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